

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

PEYTON ROUS, M.D.

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PEYTON ROOS, M.D.

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STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

III. A SECOND TYPE OF SALMONELLA NATURALLY APPEARING IN THE ENDEMIC STAGE.

By JOHN B. NELSON, PH.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 18, 1927.)

The course of a natural outbreak of paratyphoid disease in a guinea pig population was outlined in a preceding paper.¹ The active stage, which occupied a period of 8 weeks in the summer of 1924, was followed by a long endemic stage characterized by sporadic deaths. A single type of *Bacillus paratyphi* was held to be the agent throughout both stages. Continued investigation showed that a second, serologically different type had come into the population during the summer of 1926. The disease was still endemic at the time. The uniformity of the guinea pig stock had not been altered, meanwhile, by the addition of animals from outside sources. Mention should be made here of a similar occurrence reported by Lynch² who described the spontaneous appearance of a second *Salmonella* type in a mouse population. Further reference to the report will be given in a subsequent paper.

Since the introduction of specific infection into the guinea pig population all animals that died from natural causes have been autopsied. In all cases a bacteriological examination, generally of the spleen alone, has been made. Previous experience had shown that the spleen yielded a positive culture, in the majority of cases, whether the animal was a carrier or in a state of active disease. Cultures that were presumptively identified as belonging to the *Salmonella* group were tested by direct agglutination with a specific antiserum. From

¹ Nelson, J. B., and Smith, T., *J. Exp. Med.*, 1927, xlv, 353.

² Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

the onset of the epidemic up to the summer of 1926, the strains isolated from active cases and from carriers had shown sufficiently uniform agglutination reactions to be regarded as individuals of one general type. One serum employed in identification agglutinated its homologous strain through a dilution of 1:51,200. With this serum the strains examined agglutinated either to the titer limit or in the next lowest dilution, 1:25,600. It is realized that conclusions based on the outcome of direct agglutination tests alone may be misleading. The serum in question, however, was tested against a comprehensive series of other members of the *Salmonella* group and found to be low in group agglutinins. Of the types examined none agglutinated in a dilution higher than 1:800. Even though the isolated strains were not subjected to a more exact analysis by absorption it is believed that the position taken as to their unity is secure.

The first culture of the second type was isolated from a breeding sow, on July 28, 1926. At autopsy the animal showed a congested spleen. Focal lesions were not visible grossly. Peyer's patches of the small intestine were prominent and likewise congested. From the spleen there was obtained a motile bacillus which produced hydrogen sulfide and which failed to ferment either lactose or saccharose. Tested with the stock antiserum it agglutinated only in low dilution, 1:400. The following month three additional spleen cultures were isolated. These gave identical findings. Two of the guinea pigs were unweaned young. Only one showed suggestive lesions, the presence of small white plaques in the cecum and a congested spleen. The third culture was from a young, weaned guinea pig which showed an enlarged spleen together with minute focal lesions.

Animal injection was resorted to as a preliminary measure in the identification of these strains.

1 cc. amounts representing a 1:4,000 dilution of an 18 hour bouillon culture were injected intraperitoneally into 350 gm. guinea pigs. A single animal was employed for each strain. After 14 days the four guinea pigs were chloroformed and autopsied. In each case the postmortem findings were typical of paratyphoid infection. There was, however, a considerable individual variation in the type and extent of the changes produced. All showed enlarged spleens partially covered by an exudative membrane. There were no foci. Two showed purulent fluid in the gall bladder accompanied in one case by scattered liver foci. The

third showed focal lesions alone, and with the fourth the liver was normal. Three showed focal lesions in the lymphoid tissue of the small intestine; while the fourth showed small white plaques in the cecum. Pure cultures of an organism possessing characters identical with those of the injected culture were obtained from the spleen in each case. It seemed evident from the above findings that the four cultures were *Salmonella* types.

Rabbits were given serial intraperitoneal injections of heated killed suspensions of two strains for the production of antisera. The two immune sera agglutinated the four cultures equally through a dilution of 1:12,800. A strain of the first type was agglutinated in low dilution only, 1:800. The absorptive capacities of the two immunizing strains as tested by reciprocal agglutinin absorption were identical. The above results in conjunction with the animal tests establish the position of the four cultures as identical strains of a second *Salmonella* type. From August on sporadic deaths due to one or the other of the two types, provisionally designated *B. paratyphi* Types I and II, have occurred. The relative distribution of the two types within the population will be considered in another paper. It may be said that 87 cases of the second type have been observed and studied during a period of 10 months.

In addition to the different agglutinative relationships of the two types of *B. paratyphi*, there was some indication that their loci of development in the animal host tended to differ. As previously noted,¹ the most constant manifestation encountered in fatal cases of the earlier type was the presence of focal lesions in the spleen together with enlargement and congestion. Focal involvement of the lymphoid tissue of the small intestine was often observed and somewhat less frequently of the liver. With the introduction of the second type of infection the number of cases showing typical focal lesions in the spleen has decreased. Thus, from the autopsy records of 50 consecutive cases of each of the two types, focal lesions were observed 29 times with Type I and 17 times with Type II. When present in the latter type, however, the focal changes showed no difference grossly from those of the earlier form of infection. In the absence of foci, congestion and slight enlargement of the spleen were commonly observed. Focal involvement of the Peyer's patches of the small intestine was also less frequent. In the liver, on the other hand, it

was more often observed than in Type I cases. Lesions in the cecum have likewise been more frequent with the Type II infection. The wall of the cecum was generally congested. At times the congestion was diffuse, involving the entire wall or extensive areas of it. At times it was circumscribed, involving only the lymphoid tissue which stood out as deep red circular patches. In addition, yellowish or yellowish white plaques varying in number and in size were present. Sometimes the plaques were located on the peritoneal surface, sometimes on the mucous surface. At times a diffuse exudate coated the mucous surface giving a dense opaque appearance. Frequently the cecal contents was fluid or semifluid.

Other gross changes noted at times in individual cases included involvement of the cervical and mesenteric lymph nodes with hypertrophy and congestion, a purulent fluid in the gall bladder, and firm adhesions binding the liver or spleen to the peritoneal wall. In several instances an acute peritonitis was encountered. These cases, which were limited to the stock, weaned guinea pigs, were marked by a tenacious exudative membrane on the surface of the liver and spleen, a seromucoid exudate coating the intestinal tract and peritoneum, and a considerable volume of turbid, mucoid fluid in the abdominal cavity.

Involvement of the genital tract was often observed in the case of adult females from the breeding cages. The uterine wall was swollen and diffusely congested. At times a thick, mucopurulent exudate was present in the lumen. At times small exudative plaques were found on the peritoneal surface of the uterus. In several instances an abscessed condition of the mammary gland was encountered. In young female guinea pigs uterine changes aside from a slight congestion were not conspicuous. Involvement of the male genital tract was rarely encountered.

The relationship of the second type of *B. paratyphi* to other members of the Salmonella group was studied by means of direct agglutination and by agglutinin absorption. An antiserum against Strain 1149, the first Type II culture isolated, was employed in the direct agglutination tests. The antigens were fresh, unheated, saline suspensions prepared from 18 hour agar cultures and were standardized to equal opacity. The agglutination limits of Salmonella types and

one strain of *B. typhi* with the Type II antiserum are given in Table I. Of the cultures employed, guinea pig Strains 1149 and 922 were Types II and I, respectively, from the present epidemic. Guinea pig Strain IV was isolated during a Boston epidemic in 1908. As noted in a previous paper,¹ it was agglutinated by a Type I serum in low dilution only. The rabbit strains were isolated during a slight outbreak among the stock rabbits in the fall of 1926. The mouse cultures were Mouse Typhoid I and II from The Rockefeller Institute in

TABLE I.
Agglutination Limits with Type I and Type II Antiserums.

	Antiserum	
	Type I	Type II
Guinea pig paratyphoid 922.....	1:51,200	1:800
" " " 1149.....	1:400	1:12,800
" " " IV.....	1:200	1:12,800
Rabbit " 5.....	1:51,200	1:400
" " 22.....	1:400	1:12,800
Mouse " I.....	1:51,200	1:800
" " II.....	1:200	1:12,800
Rat " V.....	1:51,200	1:800
Calf " I.....	1:400	1:12,800
Swine " IV.....	1:200	1:12,800
<i>B. paratyphi</i> a Schottmüller.....	1:200	1:800
" " b Rowland.....	1:200	1:6,400
" " aertrycke 387.....	1:200	1:12,800
" <i>enteritidis</i> Gaertner (Kral).....	1:400	1:400
" <i>cholerae suis</i> X.....	1:100	No agglutination at 1:100
" <i>typhi</i> X.....	1:3,200	1:3,200

New York. The Rowland strain of *B. paratyphi* b was obtained through the courtesy of Miss Georgia Cooper of the New York City Department of Health. The other cultures were selected from the departmental collection.

The Type II serum agglutinated a number of the miscellaneous *Salmonella* cultures to the titer limit. Both of the serums were high in group agglutinins for *B. typhi*, while both were low for *B. enteritidis* and *B. cholerae suis*. The level of group agglutination for the opposite

type was somewhat higher in the case of the Type II serum. In the routine examination of cultures from positive cases of paratyphoid the limit of group agglutination of the Type II serum with the opposite type, however, varied from 1:200 to 1:800. The *Salmonella* cultures which showed a high agglutination with the Type II serum were selected for further identification by agglutinin absorption. In each case the serum in a dilution of 1:25 was absorbed twice with a 1:10 volume of packed and washed cells. The absorption was carried out at 37°C. for 5 hours, followed by approximately 16 hours at ice box temperature. The absorbed serum was finally tested in a two-fold dilution series ranging from 1:50 through 1:25,600. The results

TABLE II.

Agglutination Limits with Type II Serum after Absorption.

Absorbing culture	Agglutination after absorption	
	Absorbing culture	Homologous culture
Guinea pig paratyphoid 1149.....		1:100
" " " IV.....	1:200	1:200
Rabbit " 22.....	1:100	1:100
Mouse " II.....	1:50	1:50
Calf " I.....	1:200	1:200
Swine " IV.....	1:100	1:1,600
<i>B. paratyphi aertrycke</i>	1:100	1:200
" b Rowland	1:50	1:6,400

of the absorption tests with the Type II serum are given in Table II. Absorption with the homologous strain lowered the agglutinin content of the serum to the same level for all the cultures.

The absorptive capacity of the guinea pig culture, Strain IV; the mouse culture, Type II; the calf culture, Strain I; and the *aertrycke* type of *B. paratyphi* corresponded very closely to that of the homologous culture. By the direct method these cultures were agglutinated in low dilution only by the Type I serum. A close relationship between them and the Type II organism is indicated. The absorptive capacity of *B. paratyphi* b was approximately half that of the homologous culture. The reduction in titer of the serum for the

Type II antigen after absorption with *B. paratyphi* b was probably due to the removal of group and not specific agglutinin. By the direct method the culture was agglutinated in high dilution. The difference in absorptive capacity of the two cultures appears sufficiently marked to establish their non-identity. The Swine IV antigen reduced the titer of the serum approximately 87 per cent as compared

TABLE III.

Agglutination Limits with Mouse II, Calf I, and B. paratyphi aertrycke Serums after Absorption.

Serum	Culture	Direct agglutination	Absorbing culture	Agglutination after absorption	Culture agglutinated
Mouse II	Mouse II	1:51,200	Mouse II	1:100	Mouse II
			" II	1:100	Guinea Pig 1149
	Guinea Pig 1149	1:51,200	Guinea Pig 1149	1:100	Mouse II
			Guinea Pig 1149	1:100	Guinea Pig 1149
Calf I	Calf I	1:51,200	Calf I	<1:50	Calf I
			" I	<1:50	Guinea Pig 1149
	Guinea Pig 1149	1:25,600	Guinea Pig 1149	<1:50	Calf I
			Guinea Pig 1149	<1:50	Guinea Pig 1149
<i>B. paratyphi aertrycke</i>	<i>B. paratyphi aertrycke</i>	1:25,600	<i>B. paratyphi aertrycke</i>	1:50	<i>B. paratyphi aertrycke</i>
			<i>B. paratyphi aertrycke</i>	1:50	Guinea Pig 1149
	Guinea Pig 1149	1:25,600	Guinea Pig 1149	1:50	<i>B. paratyphi aertrycke</i>
			Guinea Pig 1149	1:50	Guinea Pig 1149

with the 99 per cent reduction by the homologous antigen. It seems necessary to assume that some specific agglutinin was removed and that the two cultures bear a relationship other than through their group agglutinin.

In order to establish the identity or close relationship of the cultures which showed nearly equal absorptive capacities reciprocal

tests were made. The Calf I, Mouse II, and *aertrycke* cultures were employed. Rabbits were immunized with the three antigens and agglutinating serums of high titer obtained. The period of immunization was longer than employed in the preparation of the Type II serum and the titer limits of the serums were correspondingly higher. Absorption was carried out, as before, with the homologous culture and with the Type II culture. The results of the absorption tests are given in Table III.

The agglutinative affinities of the second organism as established by reciprocal absorption relate it to the *aertrycke* type of *B. paratyphi*. The identity of the Type II culture, the Mouse II culture, and the Calf I culture is similarly indicated. The identity of the Type II culture, the Guinea Pig IV culture, and the Rabbit 22 culture, while unconfirmed, is suggested. The Swine IV culture appears to be a less closely related organism, but bearing some specific agglutinin in common with it. The second organism is related to the Type I strains, *B. enteritidis*, *B. paratyphi* a and b only through group agglutinin. The content of group agglutinin is low except for the latter organism from which it is differentiated only by agglutinin absorption. It also bears group agglutinin in common with *B. typhi*. The indicated relationship of the Guinea Pig II, Mouse II, and *B. paratyphi aertrycke* strains is in agreement with the work of Webster³ and of Edwards and Rettger.⁴

SUMMARY.

The spontaneous appearance of a second paratyphoid infection in a guinea pig population during the endemic stage of an earlier epidemic is reported. A comparative study of the gross pathology of the two infections indicated a difference in the loci of development of the respective organisms in the animal host. The two types were readily differentiated by direct agglutination with specific immune serums. From its agglutinative affinities the second organism was judged to be an *aertrycke* type of *B. paratyphi*.

³ Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

⁴ Edwards, P. R., and Rettger, L. F., *J. Bact.*, 1927, xiii, 73.

VARIATIONS IN THE SCOURS TYPE OF *BACILLUS COLI* FROM THE STANDPOINT OF BACTERIOPHAGIC ACTION.

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Certain strains of the scours type of *Bacillus coli*, described by T. Smith and Bryant,¹ are subject to variation, on solid media, characterized by the appearance of indented areas at the periphery of the colonies. These areas may be v-shaped or hemispherical and are underlaid by a secondary growth which differs from the primary, mucoid, opaque form in that it is non-mucoid and translucent. The bacteria comprising it are actively motile whereas those of the parent colony are non-motile or sluggishly so. Both forms produce a diffuse turbidity in bouillon. Subcultures made from the secondary growth are pure with respect to the characters enumerated and continue to breed true in subsequent generations. Subcultures from the parent colony are unstable and subject to variation under certain conditions.

Hadley² describes a somewhat similar type of variation occurring in colonies of Friedländer's pneumobacillus and of various intestinal bacteria marked by the appearance of peripheral, bluish, translucent invaginations. These may extend radially to produce a fringe or backward into the colony which may eventually be wholly consumed. The secondary growth is comprised largely of the R type of culture and upon subculture yields thin, irregular, translucent colonies. This form of variation, termed by Hadley marginal dissociation, may occur suddenly and spontaneously in cultures which have previously given a normal growth on solid media. Its analogy with the present form of variation appears close but not complete. With the colonies of the

¹ Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, xlv. 133.

² Hadley, P., *J. Infect. Dis.*, 1927, xl, 1.

scours type of *B. coli* the peripheral indentations are always present in the initial culture, made directly from the calf. They are to be observed in the majority of the colonies. The exceptions, which appear normal in that generation, invariably undergo variation upon subculture. The secondary growth, unlike that described by Hadley, has not shown characters which would suggest the R form of culture, at least when first isolated. The colonies do not present an irregular border and do not flocculate in liquid media. There is an indication, however, that the secondary growth may readily undergo partial transformation to the rough type which flocculates spontaneously and yields an irregular colony.

d'Hérelle³ has intimated that all fixed bacterial mutations are produced through the action of bacteriophage. The changes which occur in the colonies of the scours organism do bear a superficial resemblance to changes found in the colonies of susceptible bacteria exposed to bacteriophagic action. The fact that they regularly recur in generation after generation is also suggestive of the influence of some transmissible agent, carried on in subcultures or generated anew with each transfer. Unlike the eroded areas of lytic colonies, however, the indentations of the scours growth tend to increase in size as the colony ages. Moreover they are sometimes delayed until the colony is relatively old. With such a relationship in view a typical strain of the scours organism has been examined for evidence which might indicate the presence of bacteriophage.

Attempts to demonstrate a transmissible principle in cultures of the primary, mucoid type, using lysis as an indicator, have regularly met with failure. Filtrates made from 6, 24, and 48 hour bouillon cultures of a typical mucoid strain, *B. coli* 223 A, possessed no demonstrable lytic action.

In practice, 1:10 and 1:100 dilutions in 5 cc. of bouillon were made from the filtrates and inoculated with 0.05 cc. amounts of 18 hour bouillon cultures picked from individual colonies of the homologous type. At 37°C. growth was normal macroscopically and microscopically through 48 hours. Plates streaked from 1:10 dilutions after 6 hours of incubation showed normal colonies comparable to those of a control plate streaked from a plain bouillon culture. Successive passage of

³ d'Hérelle, F., The bacteriophage and its behavior, translated by Smith, G. H., Baltimore, 1926.

the mucoid type through a filtrate likewise failed to elicit any demonstrable lytic action. The filtrate of a 48 hour bouillon culture of *B. coli* 223 A, diluted 1:10 with bouillon, was inoculated with 0.05 cc. of an 18 hour culture of the same type. After 48 hours at 37°C. the culture was filtered, the filtrate again diluted 1:10, and reinoculated. This procedure was continued for six passages. The final filtrate was tested, as before, by low serial dilution in bouillon seeded with equal amounts of a young culture of 223A. Growth was normal in the dilution tubes. There was no inhibition, no macroscopic alteration in the nature of the growth, and no microscopic change in the morphology of individual organisms. Plates streaked from the 1:10 dilution likewise showed normal growth. Both the original and passage filtrates were also inactive for the non-mucoid variant.

The association of a bacteriophage with the non-mucoid variant seemed less probable. However, 24 and 48 hour filtrates of the variant and later a single filtrate subjected to serial passage were tested in low dilution against the homologous type and also against the mucoid type. Growth was followed at intervals through 48 hours and plates were streaked after 6 hours. No inhibition of growth and no change in the character of growth were observed with either type. There was no indication that the variant, non-mucoid form of *B. coli* 223 was lysogenic.

The exposure of a susceptible organism to bacteriophagic action is frequently followed by the appearance of a secondary growth which is relatively resistant to lysis. The variant form of the organism under discussion might represent such a secondary growth arising in response to the action of a lytic agent. If such were the case it should possess some degree of resistance to bacteriophagic action while the parent, mucoid form should be susceptible. An effort was made to recover an active lytic agent, from the animal host, as an indicator of the susceptibility of the two culture types.

Fecal samples were obtained by swab from a series of ten calves. Two individuals, Nos. 3 and 4, were scouring when the samples were taken. The remainder were recovered cases from which feces were secured within a week following the disappearance of active symptoms. Heavy suspensions were made from the swabs in approximately 15 cc. of bouillon and incubated at 37°C. After 3 to 6 hours an agar plate was streaked from each culture. After 48 hours they were filtered through paper and a Berkefeld N candle. The filtrates were tested for lytic activity against the A and E types of *B. coli* 223 and against at least one strain, presumptively identified as *B. coli*, isolated from the corresponding plate. Growth was observed in 1:10 and 1:100 bouillon dilutions of each filtrate and on

agar plates streaked from them. The activity of the individual filtrates against the three strains is given in Table I.

Only one sample from each calf was examined and a second passage of the cultures was not attempted with individual filtrates which showed no initial activity. Hence the results indicate only roughly the distribution of bacteriophage within the series. The filtrate from Calf 1 was selected for further study. Preliminary examination had failed to indicate any difference in the strength of the two filtrates which were active for the scours type.

TABLE I.
Action of Calf Filtrates on B. coli Strains.

Calf No.	Type of <i>B. coli</i>		
	223 A	223 E	Fecal culture
1	—	+	+
2	—	—	—
3	—	—	+
4	—	—	+
5	—	—	+
6	—	—	—
7	—	—	—
8	—	—	+
9	—	+	—
10	—	—	—

The above filtrate was retested against the non-mucoid E type of *B. coli* 223. Dilutions ranging from 10^{-1} through 10^{-12} were made in 5 cc. amounts of bouillon and inoculated with 0.05 cc. of an 18 hour bouillon culture. A culture control was included. Separate pipettes were employed throughout for each mixing. After 1 hour at 37°C . there was a faint, diffuse turbidity in all tubes. Microscopically, by hanging drop, the first two dilutions showed numerous small clumps together with motile free forms. After 2 hours there was a fine, macroscopic flocculation in the first two tubes. Microscopically there was an increase in the size and number of clumps. In addition, many individual bacteria, both clumped and free, were definitely increased in size. After 6 hours the supernatants of the first two dilutions were nearly clear, a few floccules remaining suspended. There was an abundant granular sediment at the base. The third dilution showed no visible flocculation but a scant granular sediment. The turbidity of the remaining dilutions and control was diffuse, with no floccules and no sediment. Microscopically the first two dilutions, after shaking, showed clumps of varying size with

comparatively few free forms. The majority of the bacteria displayed a swollen appearance with an increase in size of from 2 to 5 or more diameters. There was a marked variation in the shape of individual cells. Spherical, elliptical, and drumstick shapes were most common. The larger forms frequently showed one or more central vacuole-like areas of different refraction from the remainder of the cell. In addition, indistinct granules were sometimes visible. The larger of the swollen forms bore a resemblance to yeast cells. Decreasing numbers of these forms and clumps were seen through dilution 10^{-5} . The remaining dilutions and the control showed no swollen forms and only an occasional small clump.

After 24 hours there was a noticeable decrease in the bulk of the sediment at the base of the first two dilution tubes, which could not be attributed simply to gravity. The supernatants showed a faint turbidity with floccules in suspension. Dilution 10^{-3} displayed a heavier sediment with a turbidity distinctly less than that of the control. With dilutions 10^{-4} , 10^{-5} , and 10^{-6} the turbidity was equal to that of the control, but there was a scant granular sediment with floccules suspended in the lower third. The remaining dilutions and the control showed a heavy, diffuse turbidity and a very scant, compact, button-like deposit quite different from that of the preceding dilutions. Microscopically the first two dilutions showed a marked decrease in the number of swollen forms. Clumps were still visible. Some showed sharply outlined bacteria but in many cases the individual units appeared indistinct, often granular in shape. Dilutions 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} showed many clumps together with free normal bacteria and an occasional swollen form. The bacteria in the remaining dilutions and the control were evenly distributed in the fields with a few small clumps and no swollen forms.

After 48 hours there was no appreciable change in the macroscopic or microscopic appearance of the first six dilutions. Dilutions 10^{-7} and 10^{-8} were slightly less turbid than the control with scant granular sediment and suspended floccules in the lower portions. The remaining dilutions and the control showed comparable turbidity with no flocculation and no change in the sediment. Dilutions 10^{-1} , 10^{-8} , 10^{-9} , 10^{-10} , and 10^{-11} were filtered through Berkefeld candles and retested in one dilution only, 10^{-1} . The four highest dilutions showed normal growth macroscopically and microscopically through 48 hours at 37°C . Dilution 10^{-8} showed a few clumps and small swollen forms after 6 hours. After 24 hours there was a decreased turbidity with granular sediment and suspended floccules. The 10^{-1} dilution faithfully reproduced the original reaction.

A portion of the same filtrate was likewise tested against the mucoid A type, of *B. coli* 223. The same method was employed but only five dilutions from 10^{-1} to 10^{-6} were included. The tubes were examined at the same intervals as before through 48 hours at 37°C . Growth was normal in all dilutions from the start and at no time showed any variation from that of the control. Swollen forms were not seen in hanging drop preparations. Plates streaked from the 10^{-1} dilution and from the control showed no significant difference in the number of colonies and no deviation from normal in their appearance. Since the original filtrate displayed no initial lytic activity for the mucoid type an attempt was made

to adapt it. Six passages of the mucoid type were carried out with 48 hour intervals between filtrations. The final filtrate showed no demonstrable lytic action against the passage type of the organism when tested as before.

Serial passage was also resorted to in attempts to increase the activity of the original filtrate for the non-mucoid E type. Six passages were made with 48 hour intervals between inoculations. The final filtrate was tested in a dilution series with a range from 10^{-1} to 10^{-12} . The lytic strength of the filtrate was not appreciably affected. It displayed no greater tendency to cause complete lysis of the homologous bacteria, with permanent inhibition of growth, than did the original filtrate. There was, however, an increase in titer. A marked reaction was shown in dilution 10^{-3} after 6 hours and a scant reaction in dilutions 10^{-9} and 10^{-10} after 48 hours. The limiting dilution was determined, as before, by filtering and retesting in low dilution after 48 hours. Fourteen additional passages were carried out with a 24 hour interval between inoculations. The final filtrate showed no significant difference in action or in titer from the previous one. Like the original filtrate it was inactive for the parent, mucoid type of culture.

DISCUSSION.

The activity of the filtrate employed in the preceding work was weak but nevertheless definite. A growing culture of the non-mucoid variant upon exposure to a low dilution of the filtrate was first agglutinated and then altered morphologically. Individual cells became markedly exaggerated in shape, size, and internal structure. By prolonged examination it was at times possible to observe the rupture of the swollen cells. There was immediate shrinkage, with a minute granule-like residue. Complete dissolution apparently did not occur in most instances. Hanging drop preparations made from 24 hour filtrate cultures showed clumps composed in part of minute, poorly staining granules, together with an occasional swollen form and sometimes clearly outlined, deeply staining rods. The factor accountable, in part or in whole, for the cellular agglutination was slightly active for bacteria killed by heat. With dead bacteria, however, the agglutination was slow, occurred only in low dilutions, and was not accompanied by any morphological alteration of the cells. The active principle of the filtrate was relatively sensitive to heat. Exposure of the undiluted filtrate to a temperature of 55°C . for 30 minutes resulted in partial inactivation. At 65°C . for 30 minutes there was complete inactivation.

The weak lytic activity of the filtrate was demonstrable only in the

presence of the non-mucoid variant type of culture. In the presence of the parent, mucoid type it was inactive. The anomalous susceptibility of the two forms of growth together with the inactivity of their filtrates is opposed to the natural association of bacteriophage with the described scours organism as the agent responsible for variation.

CONCLUSIONS.

1. A lytic agent was not demonstrable in culture filtrates of either the parent or variant type of the scours organism.

2. The parent type was resistant to the action of a "weak" bacteriophage, obtained from the animal host, while the variant type was susceptible.

3. Exposure of the variant type to the scours bacteriophage was attended by agglutination of the cells, marked swelling, and an alteration of the contents prior to lysis.

4. The manifestations of variation which regularly occur on agar plate cultures of the scours organism do not appear to be the result of bacteriophagic stimulation.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

XII. THE EFFECT OF DIET ON HOST RESISTANCE.

FURTHER STUDIES.

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In a previous paper we described experiments in which it was shown that mice of the Rockefeller Institute strain, when fed on a McCollum "complete" diet, were far more resistant to *per os* infection with a mouse paratyphoid bacillus than were mice of the same strain fed on the routine bread and milk diet.¹ Since this paper was published, we have had it in mind to analyze the results in order to find out, if possible, which constituents of the McCollum diet were chiefly responsible for the increased resistance to infection. This work has been done during the past year, and we have sought to ascertain (1) the constituents of the McCollum diet which promote resistance to the infection, and (2) whether the observed seasonal fluctuations in resistance to the infection^{2,3} are correlated with seasonal changes in diet.

The first experiments were planned to determine (a) the effect of the omission of the butter fat from the McCollum diet, and (b) the effect of the constituents of that diet, other than butter fat, when added singly to the ordinary bread and milk ration.

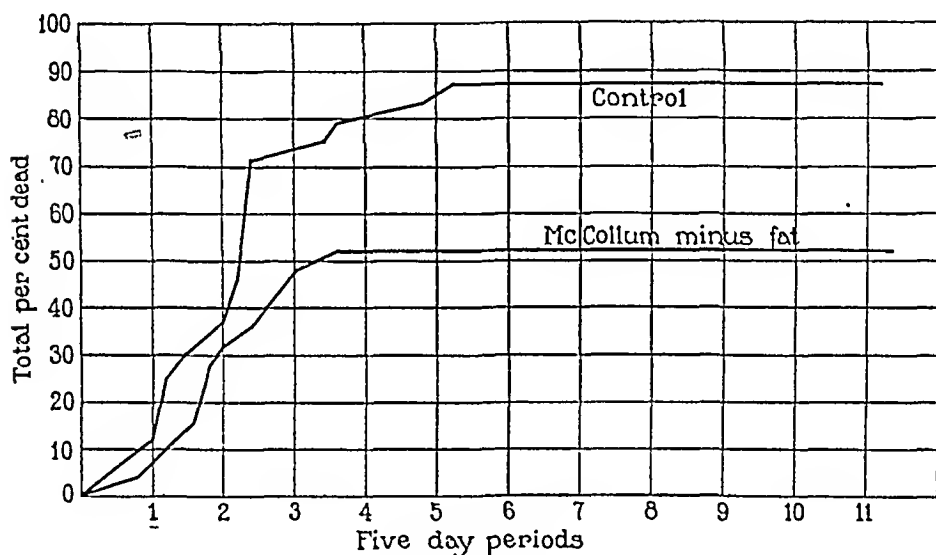
The mice employed were obtained from the Rockefeller Institute breeding room and had been reared on a diet of bread and milk, grain mixture, and dog biscuit,

¹ Webster, L. T., and Pritchett, I. W., *J. Exp. Med.*, 1924, xl, 397.

² Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 209.

³ Pritchett, I. W., *J. Exp. Med.*, 1926, xliii, 173.

from the time of weaning until they were 6 to 8 weeks of age. They were then removed to another building and placed in metal cages in groups of 12 and 13. At this time the various modified diet groups were arranged, a control group being maintained on the ordinary bread and milk diet. The mice were given the same care that they received in the breeding room; the cages were kept as clean as possible, uneaten portions of the ration being removed at the end of the day. On the day of inoculation each mouse was placed in a separate battery jar containing clean shavings and received by means of a stomach tube about 5,000,000 bacilli from an 18 hour broth culture of mouse typhoid (*B. pestis caviæ*). Each test was allowed to run 8 weeks.



TEXT-FIG. 1.

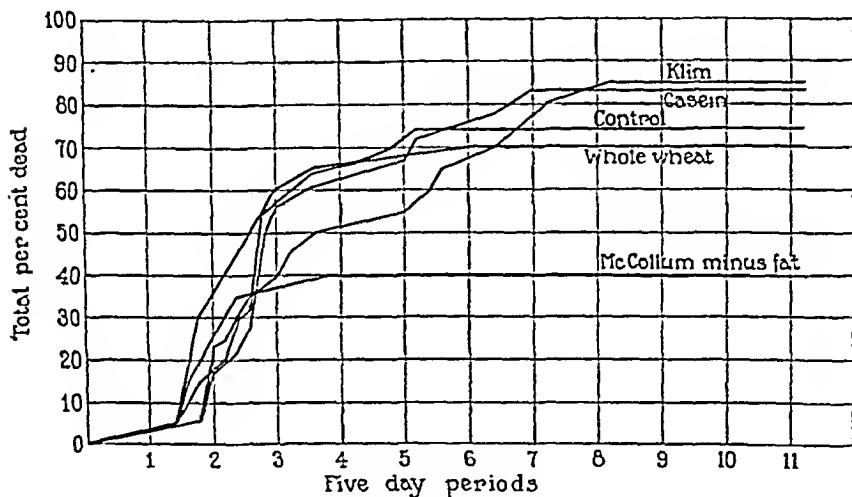
EXPERIMENTAL.

Experiment 1.—Two groups of mice of the Institute strain, one lot of 24 and another of 25, were caged in groups of 12 and 13. One lot, consisting of 25 mice, received, in addition to a constant supply of fresh water, a McCollum diet from which the 5 per cent of butter fat had been omitted, the rest of the formula being the same as that previously employed:

Whole wheat flour.	67.5 per cent.
Casein (commercial).....	15.0 " "
Milk powder (Klim).....	10.0 " "
Sodium chloride.	1.0 " "
Calcium carbonate.....	1.5 " "

The second lot, consisting of 24 mice, was fed on the bread and milk diet and served as controls. After having been on these diets for a period of 10 days, each mouse was given by means of a stomach tube the standard dose of about 5,000,000 mouse typhoid bacilli.

The mortality curves for these tests are given in Text-fig. 1. From them it will be seen that the death rate of the McCollum group is lower than that of the bread and milk group, though not so low as were the rates previously observed when the complete McCollum formula, including the butter fat, was fed.¹



TEXT-FIG. 2.

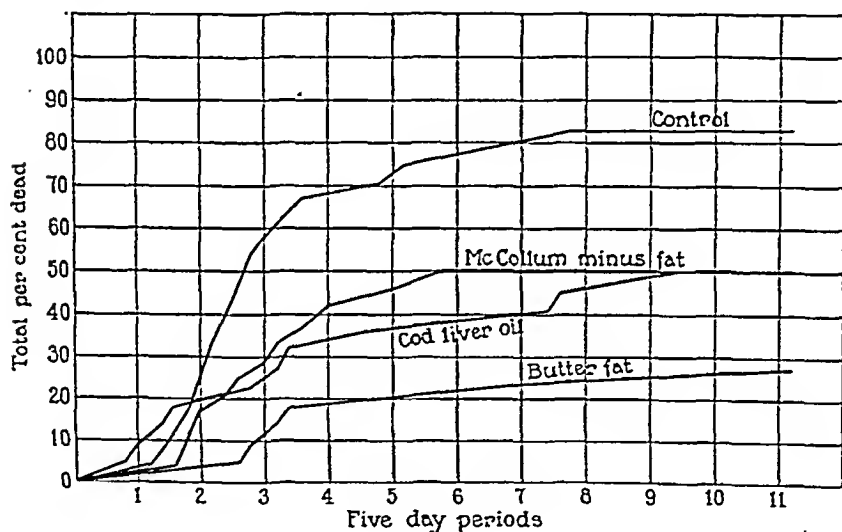
Experiment 2.—This test was planned to control Experiment 1 and in addition to try the effect of the various constituents of the McCollum diet, other than butter fat, when added separately to the routine bread and milk diet. The following diet groups were arranged, the substances used in the modified diets being thoroughly mixed with the bread and milk ration before feeding.

- A. 20 Institute mice. Bread and milk diet plus 10 per cent powdered whole milk (Klim).
- B. 18 Institute mice. Bread and milk diet plus 10 per cent commercial casein.
- C. 20 " " " " " " 10 " " whole wheat flour.
- D. 47 " " " " " " only—controls.
- E. 20 " " McCollum diet minus butter fat.

The mice were kept as before in small groups in cages. After 2 weeks on the various modified diets, each mouse was placed in a separate battery jar and re-

changes in diet, we employed for these experiments (a) a fat known to vary seasonally in its content of accessory food factors, (b) a fat known to be relatively constant in its content of such factors, and (c) a fat thought to be free of vitamins. They were represented by the following fats and oils:

1. Butter fat (from Borden's sweet butter). Subject to seasonal fluctuations in its content of fat-soluble vitamin.
2. Cod liver oil (Harris). A concentrated and relatively uniform source of fat-soluble vitamin.
3. Crisco. A hydrogenated vegetable oil thought to be lacking in vitamins.



TEXT-FIG. 3.

In addition two other diets were employed:

4. A bread and milk diet in which the milk had been subjected to the direct light of a small mercury vapor lamp, for 1 hour.
5. McCollum diet minus fat.

Experiments with several of these diets were carried out throughout the first half of the year 1926, from January to June inclusive, since previous studies had indicated that at this time of year our mortality rates were likely to be at their highest.^{2,3} The milk employed

TABLE

Experiment started	Diet	Number of mice	Number of mice															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1.15.26	Bread and milk + 10% butter fat.....	25								2	2	1	2	1				
	McCollum minus butter fat.....	24				1		1		3	1	1	1	1				
	Bread and milk—control.....	24						1		3	1	1	4	1	1			
2.16.26	Bread and milk + 5% butter fat.....	20								1	2	2		3				
	Bread and milk + 5% cod liver oil.....	20							1		2	1						
	Bread and milk + 5% Crisco.....	20								2		1	1	2				
	McCollum minus butter fat.....	20					1			2	1	1					1	
	Bread and milk—control.....	18								1	3	2	2	3	1			
3.16.26	Bread and milk + 5% butter fat.....	30				2		1	1		1	1			2	1	1	
	Bread and milk + 5% cod liver oil.....	29						1	2	2	3							
	Bread and milk + 5% Crisco.....	29				1			4	1	1				1			
	Bread and milk—control.....	31					1				2	1	2	1	4	1		
4.15.26	Bread and milk + 5% butter fat.....	29						1		1	1	3	2	1	2	1	1	
	Bread and milk + 5% cod liver oil.....	30						1			3	1	2	1				
	Bread and milk + 5% Crisco.....	29									1		3			2		
	Bread with rayed milk.....	30								2	2	2	1	1		1		1
	Bread and milk—control.....	30					2			2	1	2	2	4		1		2
5.14.26	Bread and milk + 5% butter fat.....	29							1		2	2		2				
	Bread and milk + 5% cod liver oil.....	30						2	1	2	1	1	2					
	Bread and milk + 5% Crisco.....	30					1			2	2	4	2	2	2			
	Bread with rayed milk.....	30							1		2	2	1			1		
	Bread and milk—control.....	30							1	3	1	1	1	1	2	2	1	
6.15.26	Bread and milk + 5% butter fat.....	30							1	2	1		1	1	1	2		2
	Bread and milk + 5% cod liver oil.....	30							1	3	1	1	2	1		1	1	
	Bread and milk + 5% Crisco.....	29							1	2		1		2	1	1	2	
	Bread with rayed milk.....	30								2	2	2				2	3	
	Bread and milk—control.....	30								2	2	2		1		2		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

L

dying each day			Total dead	
			Number	Per cent
1	19	20	12	48
	21	22	11	46
	23	24	21	87
	25	26	8	40
	27	28	8	40
	29	30	10	50
	31	32	8	40
	33	34	14	78
	35	36	14	47
	37	38	12	41
	39	40	15	52
	41	42	16	52
	43	44	17	59
	45	46	16	53
	47	48	9	31
	49	50	13	43
	51	52	19	63
	53	54	12	41
	55	56	11	37
2	1	2	23	77
	3	4	14	47
	5	6	19	63
	7	8	17	57
	9	10	14	47
	11	12	16	55
	13	14	14	47
	15	16	17	57
	17	18	16	55
	19	20	14	47
	21	22	17	57
	23	24	16	55
	25	26	14	47
	27	28	17	57
	29	30	16	55
	31	32	14	47
	33	34	17	57
	35	36	16	55
	37	38	14	47
	39	40	17	57
	41	42	16	55
	43	44	14	47
	45	46	17	57
	47	48	16	55
	49	50	14	47
	51	52	17	57
	53	54	16	55
	55	56	14	47
3	1	2	17	57
	3	4	16	55
	5	6	14	47
	7	8	17	57
	9	10	16	55
	11	12	14	47
	13	14	17	57
	15	16	16	55
	17	18	14	47
	19	20	17	57
	21	22	16	55
	23	24	14	47
	25	26	17	57
	27	28	16	55
	29	30	14	47
	31	32	17	57
	33	34	16	55
	35	36	14	47
	37	38	17	57
	39	40	16	55
	41	42	14	47
	43	44	17	57
	45	46	16	55
	47	48	14	47
	49	50	17	57
	51	52	16	55
	53	54	14	47
	55	56	17	57

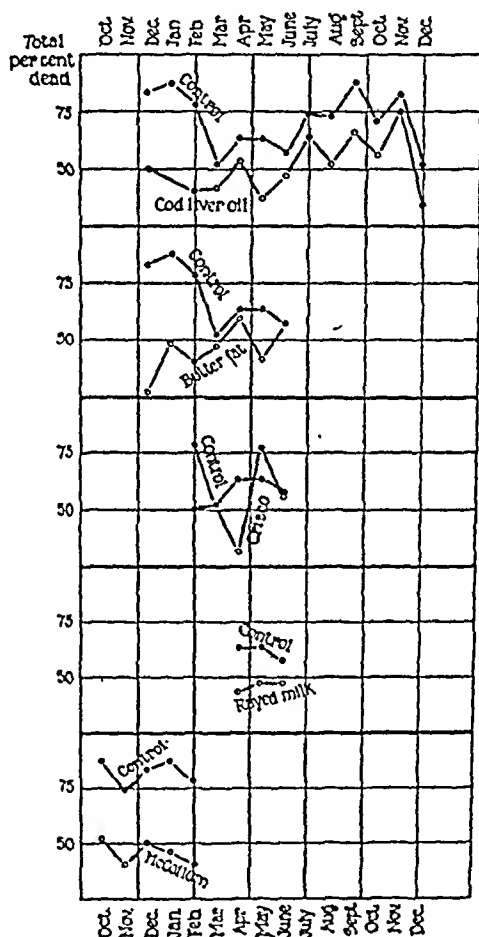
TABLE

Experiment started	Diet	Number of mice	Number of mice														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
7.27.26	Bread and milk + 5% cod liver oil.	50							1	2	2	1	3	3	1	1	2
	Bread and milk—control.	50							1	2	2	2	2	1	3	1	2
8.27.26	Bread and milk + 5% cod liver oil.	50								1	3		4	1	5	1	
	Bread and milk—control.	48						1	2	2	1	2	3	1	6	3	
9.22.26	Bread and milk + 5% cod liver oil.	50						1		4	1	5	2	3	2		
	Bread and milk—control.	50						2	1	1	3	7		16	3		1
10.20.26	Bread and milk + 5% cod liver oil.	48						1	2		5	3		1	4		2
	Bread and milk—control.	48					2	1			1		1	2	5		3
11.16.26	Bread and milk + 5% cod liver oil.	48							1	2	3	2	1	2	7	6	
	Bread and milk—control.	49						3	1		3	2	5	4	15	3	2
12.15.26	Bread and milk + 5% cod liver oil.	50								1		2	2			3	
	Bread and milk—control.	50									3	1	7	1	1	1	2
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

—Concluded.

dying each day																																	Total dead							
20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	Number	Per cent		
1			2	1	1	1		3							1						1	1															32	64		
1	1	1		1		2			2	1					6						1	3										1		1			37	74		
1				1													1					2		1				1						1	1			26	52	
	7			1							1										1	2							1						1			35	73	
3	1	3			1				1	1										1		1															33	66		
1	2			1	1					2							1				1																	44	88	
3				1		1															1				1				1									27	56	
4	1	1				2	1	1						3							2	2								1								34	71	
	2	1	1	1	1			1	2			1		1																								36	75	
																			1																			40	82	
1			1		1								2															2			1							17	34	
1										1					1											4	1							1					26	52
19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56			

for Diet 4 above was identical with that used for the control bread and milk diet except that it had been exposed for 1 hour, in a shallow glass vessel, at a distance of 4 or 5 inches, to the light of a small mercury vapor lamp, and was thoroughly stirred with a pipette at the end of the first half-hour. From the month of March on the modified Mc-



TEXT-FIG. 4.

Collum diet (minus butter fat) was abandoned, and only the bread and milk control diet together with its fat and rayed milk variants was employed. This was done in an effort to simplify the problem as much as possible and to permit the use of larger numbers of mice in each diet group. From February on only 5 per cent of fat was used in the

modified diets, instead of the 10 per cent employed in the earlier experiments. This was done in order that the fat content of the modified bread and milk diets might be more nearly comparable to that of the whole McCollum diet, the original formula for which, as given us by Dr. E. V. McCollum, called for 5 per cent of butter fat. The results of all these experiments are condensed in Table I and in Text-figs. 4 and 5.

Text-fig. 4 shows the monthly mortality rate for each of the modified diet groups, and for the corresponding control groups, from October, 1925, to December, 1926, inclusive, thereby including all the experiments recorded in this paper. The mortality rate for any one month represents the total mortality of the mice used in the experiment begun in that month, all experiments being started on or near the 15th. Although the points on this chart are discontinuous, related points have been roughly connected into curves, in order to enable the reader to compare more easily the mortality rates of the mice on the various modified diets with the rate of the controls on the plain bread and milk diet. The total per cent mortality is indicated along the ordinate, while the successive months are arranged along the abscissa. In each division of the chart appear two curves—that of the diet group indicated, together with that of the control group for the same period. In October and November only the McCollum diet minus fat was used, in addition to the control bread and milk diet. From December on, several fat diets were included in the experiment and for each one a separate curve is plotted, in each case accompanied by the corresponding portion of the control curve. These diets were continued through June, 1926, after which time only the cod liver oil and the control diet groups were employed.

It will be seen that, almost without exception, the total mortality rate of the control group was higher than that of the groups on the various modified diets. The results presented here are somewhat fragmentary, since only the cod liver oil and control series were carried on throughout the year. Nevertheless, within the longer or shorter periods covered, the effects of all the modified diet groups, save one, were consistent. In every case except that of the group receiving the bread and milk diet plus 5 per cent Crisco, the mortality rate is consistently lower than in the control group on the unmodified bread and milk diet. They follow with considerable regularity the fluctuations

in mortality of the control group, a tendency to stabilization of the death rate at a level lower than that of the controls being indicated. The mortality rate of the Crisco series, on the other hand, was three times lower than, once higher than, and once equal to that of the control series.

Text-fig. 5, like Text-fig. 4, is compiled from the data given in Table I, and shows the separate mortality curves of each of the monthly diet experiments. In each division of the chart appear two curves, plotted as total mortality against time—that of the diet group indicated along the left margin of the chart together with the corresponding control curve. The curve for the control group in any one month is therefore repeated as many times as there were modified diet groups in the experiment for that month. In this chart it is easy to compare the separate diet curves throughout their course with the corresponding control curves. It will be seen that for the most part the various diet curves exhibit considerable similarity in their tendency to approximate or diverge from the corresponding control curve in any given month. In December, January, and February, the final mortality rate of all the modified diet groups was considerably below that of the controls. In March, however, the mortality rate in the control group dropped and closely approached the level usually attained by the groups on the modified diets. In April and May, the death rate in the control group increased somewhat and again diverged from the level usually attained by the modified diet groups; the only decided exception is in the May group receiving the modified diet plus Crisco, which showed a mortality rate above that of the May control group. In June, the mortality rate in the control group again dropped and approximated the level maintained, with fair regularity, by the modified diet groups.

In the period from July to December inclusive, only the control diet and the cod liver oil diet were employed, 50 mice being included monthly in each diet group. In July, both curves were surprisingly high for this time of year and closely approximated each other. Thereafter they showed the customary divergence, except in the month of November, when both were again high.

It seems to be clearly indicated by these experiments that butter fat is the most important single constituent of the McCollum diet in

protecting the mice against *per os* infection with the bacillus of mouse typhoid. The degree of protection afforded by butter fat, when added to the control diet, was about equal to that of all the other constituents of the McCollum diet combined, and was closely approximated by that of cod liver oil.

While seasonal fluctuations in resistance were not completely eliminated by the various modified diets, they were reduced. There was apparently a tendency for the various modified diets to stabilize the death rate at a point lower than that usually reached by the mice on the control diet.

DISCUSSION.

Recently a paper has appeared by Vaile¹ on the correlation between fat consumption and susceptibility to tuberculosis. Through a period of 7 years' country practice in England, he studied various families in which cases of tuberculosis had occurred, dividing up their members into two groups according to whether they were or were not normal fat eaters. He concluded that "when several persons are equally exposed to infection [with tuberculosis], those who habitually eat animal fat will probably escape—but may not, while those who avoid fat may escape—but probably will not." He could not see that forcing fat consumption above the level reached by the normal fat eater was profitable.

So far as I am aware, most of the published experiments on the effect of diet upon susceptibility to disease are concerned primarily with diets in which one or more of the accessory food substances is markedly deficient or wholly lacking. In the experiments here recorded, this was not the case. The "control" diet employed here was one which had been shown, through a period of years, to be adequate for the breeding and rearing of successive generations of mice, apparently without any loss of vigor. Though somewhat fragmentary, the experiments here recorded do suggest, however, that the addition of 5 per cent of an active animal fat to an apparently adequate diet can so improve its quality as to increase the resistance of mice fed on such a modified diet to *per os* infection with the bacillus of mouse typhoid. The reason for the beneficial effect of these modifications of our bread

¹ Vaile, W. B., *Lancet*, 1927, ccxii, 72.

and milk diet, aside from the probable promotion of better general health, is not known. The bread and milk food may be one of the borderline diets referred to by Cramer,⁵ who observes that the deficiencies of an apparently adequate diet may manifest themselves only when the animal is subjected to a strain. It is difficult to say whether these deficiencies could be made good in a scant 2 weeks' feeding on the modified diets, before inoculation with the bacillus of mouse typhoid. The consistency of the results would seem to indicate, however, that the apparently increased resistance of the mice on the modified bread and milk diets containing butter fat and cod liver oil, as well as those on the modified McCollum diet, was not due to chance.

SUMMARY.

When 5 per cent of butter fat or cod liver oil is added to a bread and milk diet, in itself adequate to promote the breeding and rearing of a healthy stock of mice through a period of years, the resistance of these mice to *per os* infection with the paratyphoid mouse typhoid bacillus (*B. pestis caviæ*), as compared to that of mice on the unmodified diet, is definitely increased. A similar effect may be obtained with a McCollum "complete" diet, even when the butter fat is omitted, and with a bread and milk diet in which the milk used has been rayed with a mercury vapor lamp. When an inactive fat like Crisco is added to the bread and milk diet, the results obtained are not very clear-cut. While seasonal fluctuations in resistance to mouse typhoid were not completely eliminated by the various modified diets, they were nevertheless reduced, the modified diets tending to stabilize the death rate at a point lower than that usually reached by the mice on the control diet.

⁵ Cramer, W., *Brit. J. Exp. Path.*, 1922, iii, 298.

AN EXPERIMENTAL STUDY OF DIATHERMY.

I. THE MEASUREMENT OF LUNG TEMPERATURE.

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INTRODUCTION.

The word "diathermy" means heating through. It was coined by Nagelschmidt (1) to describe the effects of the passage of certain electrical currents through the human body. Tesla¹ (2) is responsible for perfecting apparatus whereby high tension alternating currents of high frequency can be produced. In the *Electrical Engineer* for December, 1891, Tesla says: "Without vouching for all the results, which must of course be determined by experience and observation, I can at least warrant the fact that heating would occur by the use of this method of subjecting the human body to bombardment of alternating currents of high potential and high frequency." He goes on to throw out the suggestion that such heating of the body may some day find an important place in therapeutics. This prediction is in a sense correct. Diathermy has come to be one of the most widely used of the so called "physiotherapeutic modalities." Moreover, a copious literature, mostly of an empirical and casuistic kind, has sprung up, dealing with the therapeutic application of diathermy in a variety of clinical conditions. Much of this prolific literature attributes the beneficent action of these currents solely to the production of local deep heat, though some writers go as far as to stress the value of "cellular massage," which they claim the current produces. Medical diathermy, as distinguished from fulguration and cauterization, is today being used in the treatment of many diseases,

¹ We have since learned that Dr. Elihu Thomson of the General Electric Company was probably the first to construct a dynamo capable of producing high frequency currents.

such as angina pectoris, arteriosclerosis, arthritis, asthma, bronchitis and bronchiectasis, cholecystitis, essential hypertension, gonorrhea, gout, hemorrhoids, impotence, intermittent claudication, lumbago, lupus, myocarditis, neuralgia, paresthesias, pneumonia, Raynaud's disease, scleroderma, sciatica, tabes, tuberculosis, uterine displacements, varicose veins, whooping cough, and xanthelasma. For the successful treatment of each one of these conditions there are one or more enthusiastic champions.

Such a list naturally gives one pause and makes for skepticism, especially as it is so extraordinarily difficult to arrive at sound judgments in regard to remedial measures. Still, it should be borne in mind that the application of heat with the production of hyperemia is a time-honored medical procedure and one which may well have unsuspected usefulness. In the treatment of pneumonia, particularly, reports of the successful application of diathermy have been numerous, widespread, and enthusiastic. Stewart's (3) work is especially suggestive. The rationale for the use of diathermy in pneumonia has been variously ascribed to the hastening of resolution, the stimulation of the normal defenses of the body, and the direct lethal action on the microorganisms of the heat developed in the lung.

We were led to a study of the bodily responses to high frequency currents as a preliminary to an investigation of the value of diathermy in pneumonia. D'Arsonval (4) made the fundamental observation that when this type of current is passed through the human body muscle contraction is felt up to about 5000 interruptions per second. At 5000 interruptions muscle contraction is feeble, disappearing beyond 10,000.² At these frequencies, however, there occurs an unpleasant feeling of heat. The problem which confronted us was to find out whether deep localized heat could actually be produced in the body. Before presenting an account of our experiments and the

² Nernst (5) developed mathematically a generalization stating that the physiological threshold for relatively weak alternating currents varies inversely as the square root of the frequency. The number of times a current must alternate if it is to lose its stimulating and electrolytic powers depends upon its strength. Strong currents must have a higher frequency than weak ones. The Nernst formula is $I = \sqrt{NC}$, where I = intensity of alternating current, N = number of complete cycles per unit of time, and C = a constant.

results obtained from them, it will be well to consider briefly the character of the so called diathermy current and some of the laws governing its use.

The Nature of the So Called Diathermy Current.

The diathermy current is an alternating current of high voltage and low amperage, very rapidly reversed in its polarity. Usually the voltage employed in the secondary circuit is approximately 30,000 to 40,000, the voltage in the body being lower, but not accurately ascertainable. The current is from 1 to 2 amperes, and the frequency of oscillation about 1,000,000 to 2,000,000 cycles per second.³ Such currents can be produced by suitable apparatus consisting essentially of the parts shown in Fig. 1.

In this diagram *b* is the core of a transformer which transforms the low voltage current in the primary coil (*a*) into a high voltage current of the same frequency in the secondary coil (*c*). This current charges the condenser (*d*) to a voltage sufficient to break down the insulation of the spark-gap (*e*). The condenser (*d*) then discharges through the circuit *d e f* with an alternating current of high frequency determined by the small capacity of (*d*) and the small inductance of (*f*). The current induces in the oscillator (*g*) a current of the same high frequency but of again increased voltage.

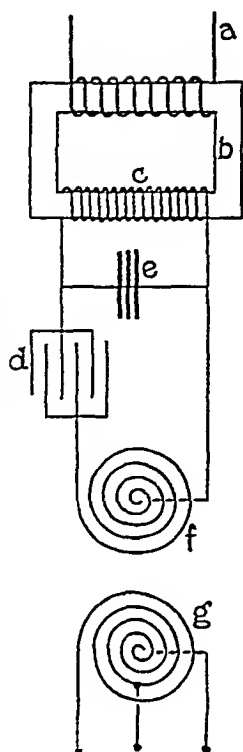


FIG. 1. Diagram of high frequency apparatus after Cumberbatch (6). (*a*) primary coil, (*b*) transformer, (*c*) secondary coil, (*d*) condenser, (*e*) spark-gap, (*f*) oscillator, (*g*) resonator.

³The physical constants of the apparatus which we used were:

Primary voltage, 110.

Secondary transformer voltage, 36,000.

Frequency in primary circuit, 60 cycles per second.

Frequency in secondary or oscillating circuit, 1,250,000 cycles per second.

Amount of capacity in secondary circuit about 900 cm.

Damping factor in secondary circuit, or logarithmic decrement, slightly below 0.5.

It is not unlikely that modern radio equipment may soon be used to advantage to replace this type of spark-gap machine.

The frequency of oscillatory discharge will depend upon: (1) the amount of self-induction in the spiral oscillator, (2) the capacity of the condenser, and, to a slight extent (3) the resistance of the circuit.

When a current traverses a conductor, heat is generated. The number of units of heat developed in the conductor is proportional to (1) its ohmic resistance, (2) the square of the current strength, (3) the duration of flow. For a continuous current the heating effect is proportional to I^2R , where I is the steady current and R the normal resistance of the conductor at any definite temperature. With diathermy high frequency currents the heat is again proportional to I^2R , but in this instance I denotes the average flow of current throughout the successive cycles, and R is the ohmic resistance plus the effect of dielectric loss.

The heating effects of these currents have for the most part been studied in non-living systems. A favorite experiment has been the coagulation of egg albumin or the cooking of meat and potatoes. Two statements are frequently made concerning the diathermy current, first, that the current always flows through the shortest path between the electrodes regardless of the resistance it encounters; second, that the heating occurs first at the center of the current path and later at the two ends. In another communication (7) we will present evidence to show that neither of these statements is strictly accurate.

Arguments from analogy based on such *in vitro* experiments have been carried over to the clinical application of diathermy, and have been responsible for many false assumptions. The living body is not a sausage, nor yet a tube filled with albumin water. It is a heterogeneous system composed of tissues with different specific conductivities and heat capacities. Moreover, these tissues are variously placed and must therefore lose heat at different rates, according to the efficiency of their circulation, and the opportunity afforded them for heat loss by radiation, convection, and conduction. Thus far, it has not been definitely established either that deep local heat can be produced by the diathermy current or, indeed, that the current penetrates into the deeper portions of the body.

Recently, Bethman and Crohn (8) have presented evidence which they believe indicates that the so called "skin effect" is a factor in keeping the current near the surface of the body. Moreover, these authors state that in experiments with anesthetized dogs they have "found it extremely difficult at any time to raise the systemic temperature more than a few fractions of a degree." Such has not been the case in our experiments, as will be brought out later, nor in the experiments reported by Lonergan (9). Dowse and Iredell (10), applying the formula developed by Lord Rayleigh, A. Russell, and others, "assume that no measurable skin effect will be present" in the passage of a current of 10^6 periods per second through the human body. They emphasize, further, the unreliability of attempting to measure current strength in the body accurately by means of a hot wire ammeter.

The fact of the passage of current through tissue can best be established by proof of heat development in the tissue. That this heat will be quickly dissipated, and that it may be conveyed to the tissue from adjoining structures, are two complicating conditions which need to be carefully controlled.

It has been the object of this study to learn whether or not local deep heat is developed in the living animal body by the passage of the current, and if so, under what conditions.

EXPERIMENTAL.

I. Effect of Diathermy Current on the Rectal Temperature.

There can be no doubt that the general systemic temperature as measured by a rectal thermometer can be raised by the passage of the diathermy current. This has been shown by Setzu (11), von Zeynek and his coworkers (12), and Lonergan (9), as well as frequently in our own experiments. In Table I it will be seen that in unanesthetized individuals of three different species the rectal temperature as observed by us was raised from 1–2°C. in as many hours.

In anesthetized animals (dogs) we have frequently seen a rise in rectal temperature of as much as 5–6°C. This increased heating effect must be due to inability to provide for heat loss, owing to partial paralysis of the heat-regulating mechanism by the anesthetic. Nor-

mally an animal will compensate for a moderate decrease or increase in the systemic temperature by increasing the heat loss or varying the metabolic rate. Under the influence of an anesthetic, however, this mechanism may be impaired, and the systemic temperature will fall rapidly unless heat loss be prevented (13). This phenomenon is well shown in the control periods of most of our experiments. Careful wrapping of the dog in blankets prevents heat loss to a fair degree and similarly assures a greater rise in systemic temperature during the passage of the current.

TABLE I.

Species	Milliamperes per sq. inch of electrode surface	Duration of current flow	Rise in rectal temperature
			°C.
Rabbit	100	2 hrs.	2.7
Dog	90	2 hrs. 13 min.	1.1
Man	66	1 hr. 21 min.	1.0

II. Effect of Diathermy Current on Deep Temperature.

(a) Intraabdominal Temperature.

Experiment D 8.—A male mongrel terrier was anesthetized by the intravenous injection of 2.97 gm. of barbital-sodium dissolved in physiological salt solution. The sides of the abdomen were shaved and lead-tin electrodes measuring 8×10 cm. were applied laterally. A mercury thermometer was inserted into the rectum, a second thermometer was placed outside of the electrical field by passing it through the abdominal wall so that its bulb lay in the abdominal cavity 5 cm. from the superior edge of the electrodes. A third thermometer was so placed that its bulb lay in the abdominal cavity at a point midway between the two centers of the parallel electrodes. A diathermy current of 1000 milliamperes (with a current density of less than 100 milliamperes per square inch) was passed through the abdomen for 1 hour. The result was a rise in rectal temperature of $1.76^{\circ}\text{C}.$, a rise of temperature in that part of the abdomen outside the electrical field of $1.76^{\circ}\text{C}.$, and a rise of temperature in the abdominal cavity between electrodes of $2.62^{\circ}\text{C}.$ The dog was then suddenly killed, while the diathermy current was flowing. After death the temperature in both rectum and upper abdominal cavity continued to rise at approximately the rate previous to death, while the temperature in the midabdomen between electrodes suddenly shot up and in the next hour showed an increment of $17.73^{\circ}\text{C}.$, or nearly seven times as great a rise as recorded during life. This latter fact is in agreement with the temperature changes observed by Lonergan

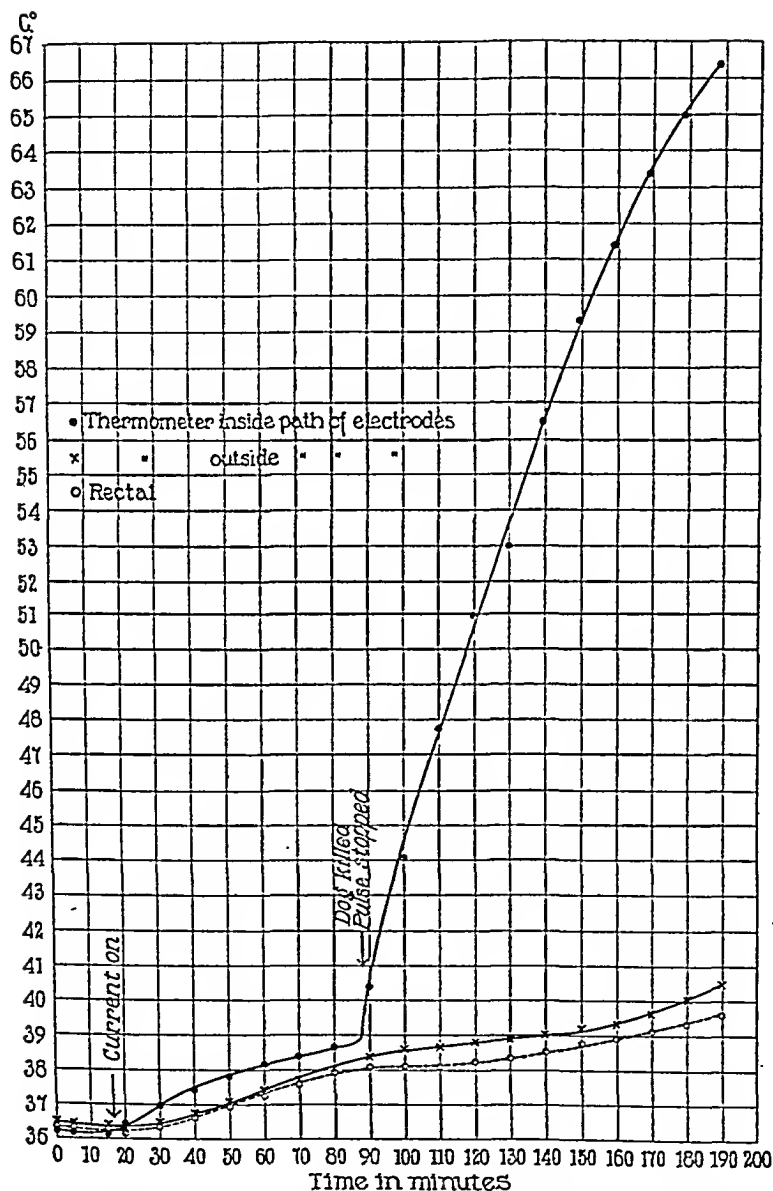


FIG. 2. Curve showing effect of death on heat recorded by thermometer placed in abdomen between electrodes.

(9) during diathermy of a dog's hip joint before and after death. The temperature changes recorded in the experiment just described (D 8) are graphically shown in Fig. 2.

This experiment brings out several points of interest. The temperature rise during life in that portion of the abdominal cavity situated between the electrodes was only a little greater than the rise in the upper abdomen and rectum. After death, however, there was a great and sudden heating up of the abdomen between the electrodes, suggesting that a compensatory cooling mechanism had ceased to operate with the cessation of life. The rectal thermometer reading here cannot be regarded as a true record of the systemic temperature because of its proximity to the heated area.

(b) Intrapleural Temperature.

In another similar experiment (D 9) in which a thermometer was inserted into the left pleural cavity between electrodes placed laterally on the shaved chest wall, the temperature during 1 hour of current flow rose by 1.88°C . with a rise in rectal temperature of 1.21°C . After death, another hour of diathermy produced an elevation in intrathoracic temperature of 11.62°C ., whereas the rectal temperature increased by only 0.08°C . Death, therefore, appears to permit a local heating in the deep tissues between electrodes which does not occur during life. It seemed fairly obvious that the interruption of normal circulation would be found accountable for this discrepancy.

To study the relation of local circulation and heat production experiments were planned in which the temperature of the lungs could be measured by thermocouples. Previous work (14) had provided us with methods for modifying and interrupting the pulmonary circulation. A description of the application of these methods to the present problem will be found in a companion paper (15).

Technique for Measuring Lung Temperature.

The accurate measurement of lung temperature in the living animal during heating by diathermy was not easy to accomplish. After discovering and ruling out a number of sources of error, we achieved a method which has proved satisfactory.

The dog is anesthetized by the intravenous injection of a solution of harhital-sodium. The sides of the thorax are shaved. Lead-tin electrodes, measuring in most instances 3×4 inches, are applied to the chest wall with the superior edge well up in the axilla. The interposition of 8 to 12 layers of gauze soaked in a solution consisting of equal parts of glycerol and saturated salt solution assures a good contact. Great care is taken to have the planes of the electrode surfaces as nearly parallel as possible in order to prevent the so called "edge effect"—*i.e.*, the current passing largely between the proximal edges of the electrodes. A convenient method for holding the electrodes in place was accomplished by encircling the thorax with two or three rubber tubes and slipping the electrodes under them. These tubes permit the free motion of the chest wall and still, by their elasticity, hold the electrodes firmly against the skin.

Thermocouples were made by twisting together $5\frac{1}{2}$ foot lengths of No. 31 gauge enamelled copper wire and double silk-covered "constantan" wire treated with one coat of shellac. These were then shellacked and allowed to dry. The two wires were threaded through the shank of a No. 20 gauge hollow Luer needle 11.5 cm. long. The exposed tips of the wires were soldered to the point of the needle in such a manner that the solder closed smoothly the bevelled opening of the needle. The wires were protected by covering them with thick walled rubber capillary tubing, the end of which was passed over the butt of the needle. The free ends of the exposed wires were connected with a galvanometer through a constant temperature junction obtained by a thermos bottle thermostat as described by Clark (16). Thermocouples such as these can be easily prepared in the laboratory. Before use they were calibrated against a Bureau of Standards thermometer which could be read to 0.02°C . When prepared from the same materials and in identical manner, the calibration curves agreed so closely that one curve could be used for all the thermocouples. The calibrations were made at known resistances and each resistance required its own curve. Resistance was supplied by an ordinary Leeds and Northrup resistance box.

The position of these thermocouple needles in respect to the electrical field was found to be of great importance. To obtain accurate temperature measurements the needles must be so placed that they cut the electrical field at right angles. In other words, they must be parallel to the planes of the electrodes, otherwise a concentration of the high frequency current occurs at the needle point, which is then heated more than the surrounding tissue. To avoid this contingency the needles were inserted into the thoracic cavity either from its anterior or posterior aspect so that the final disposition of electrodes and thermocouples was as shown in Fig. 3.

In all experiments it was made certain that turning the current on or off caused no sudden fling in the galvanometer due to concentration of current at the thermocouple. The error introduced by heat loss due to conduction through the needle shank seems a negligible one in view of the very close agreements between temperature so measured

and temperature measured by Bureau of Standards thermometers inserted in the rectum. That this source of error was negligible was shown by heating a portion of the needle shank which lay under the skin or projected outside the body wall. This caused no change in the galvanometer reading, showing that sufficient heat to cause an error in reading was not conducted along the needle to the thermocouple.

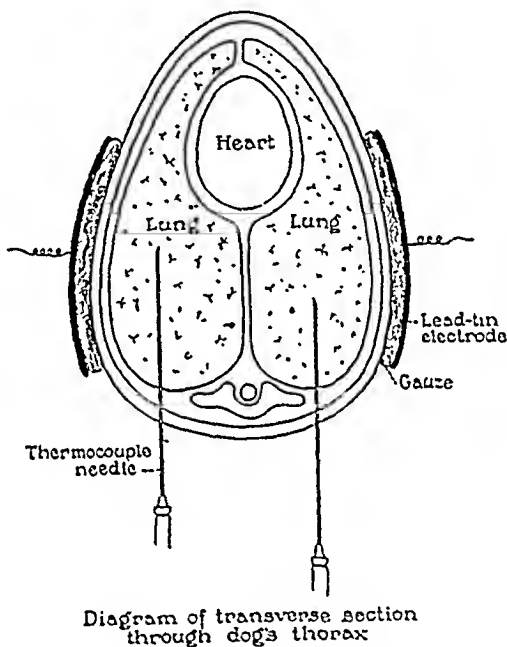


FIG. 3. Diagram of transverse section through a dog's thorax, showing relative positions of electrodes and thermocouples.

(c) *Intrapulmonary Temperature.*

Experiment D 12.—An experiment in which we used the general technique just described was performed on a male American bulldog weighing 14.7 kilos. Thermocouple needles were inserted into the right lower lobe, the right ventral lobe, and the left lower lobe. The position of the thermocouples was verified at the close of the experiment. Simultaneously the rectal temperature was recorded with a mercury thermometer. The average of the three lung temperature readings which agreed within $\pm 0.05^{\circ}\text{C}.$ before the current was turned on was $0.39^{\circ}\text{C}.$ below the rectal temperature. During diathermy the lung temperature rose slightly above the rectal, not exceeding it by more than $0.4^{\circ}\text{C}.$ Toward the close of the experiment,

after 3 hours of diathermy, this difference was even less. The temperatures are shown graphically in Fig. 4.

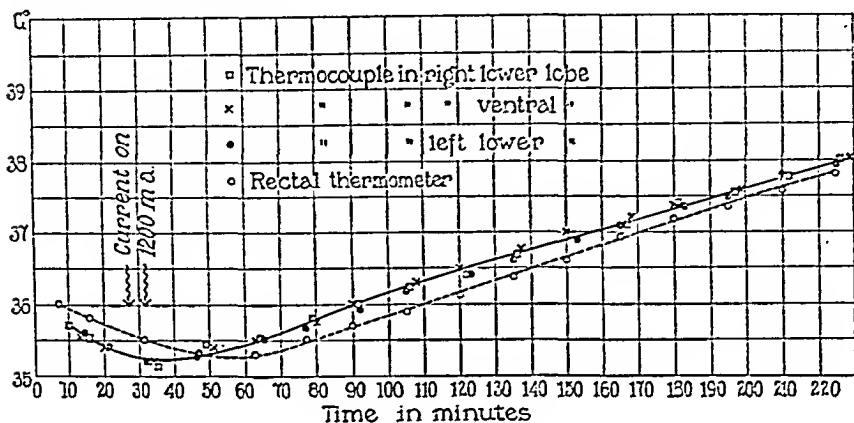


FIG. 4.

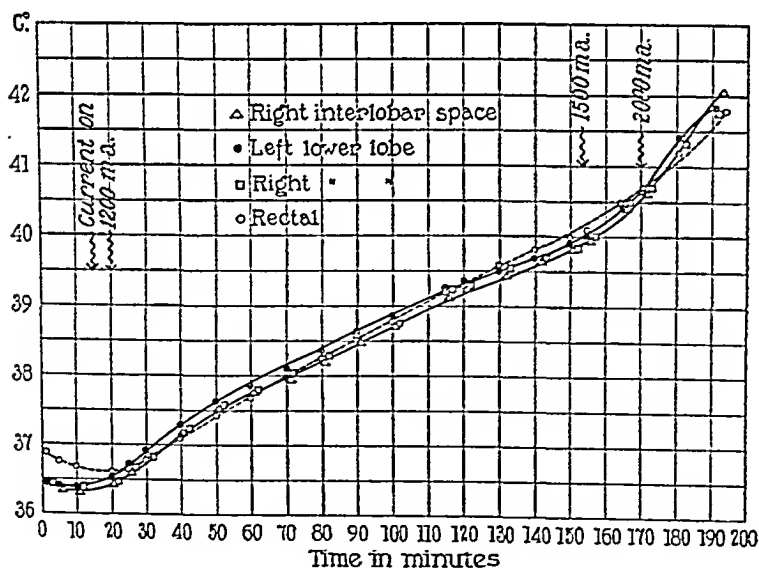


FIG. 5.

FIGS. 4 and 5. Curves showing temperatures recorded by thermocouples in the lungs and by rectal thermometers, in anesthetized dogs subjected to high frequency currents.

Experiment D 11.—This experiment was repeated on another dog. Three thermocouples were again inserted into the thoracic cavity. One of them lodged in the left lower lobe, one in the right lower lobe, and one lay between the right ventral and lower lobes in contact with the visceral pleura. The readings on these three thermocouples seldom varied by more than 0.1°C . Before turning on the current the three readings were: left lower lobe 36.45°C ., right lower lobe 36.43°C ., right visceral pleura 36.42°C . At this time the rectal temperature measured by a mercury thermometer was 36.88°C . After $2\frac{1}{2}$ hours of diathermy, the lung temperatures had risen respectively to 41.43°C ., 41.35°C ., and 41.43°C ., while the rectal temperature was 41.21°C . Fig. 5 shows the lung and rectal temperatures in graphic form.

This kind of experiment has been done repeatedly, and in some cases rectal thermocouples were used in conjunction with rectal thermometers. The same type of curve was always obtained.

DISCUSSION.

Examination of Figs. 4 and 5 will show that whereas the lung temperature in the anesthetized dog normally lies 0.3 – 0.4°C . below the rectal temperature, this relationship is reversed during the passage of high frequency currents of strengths equivalent to those usually employed in therapy. The lung temperature now exceeds the rectal temperature, but only by a few tenths of a degree. Moreover, there is a tendency for the lung to cool and gradually to approximate the rectal temperature again unless the current strength is augmented. This is well shown in Fig. 4, where the lung temperature crosses the rectal temperature first at the start of current flow, gradually falling below it, but again crosses it when the milliamperage is increased. In the light of subsequent experiments this gradual fall in lung temperature may be interpreted as resulting from increased blood flow through the lung and increased pulmonary ventilation.

The fact that death allows a sudden increase of heat in the deep tissue between the electrodes, and that this occurs in the abdominal organs as well as in the thoracic, suggests that the circulation rather than the respiration is chiefly responsible for the removal of heat from the lungs. Whether or not the heating of deep tissues is the result of the passage of current through them or is due to conduction from the more superficial structures has not yet been established. This question will be considered in another communication.

SUMMARY AND CONCLUSIONS.

1. Experimental evidence is furnished to show that in normal animals the rectal temperature can be elevated by the passage of high frequency currents.

2. During life the intraabdominal and intrathoracic temperatures can be increased only slightly above the rectal temperature.

3. The lung temperature in the anesthetized dog normally lies 0.3–0.4°C. below the rectal temperature. During the passage of diathermy currents of strengths equivalent to those used in therapy this relationship is reversed—the lung temperature exceeding the rectal temperature by about the same value.

4. Immediately after death, the temperature rises abruptly in the deep tissues between the electrodes.

5. For the measurement of deep temperature special thermocouples have been devised. Their method of preparation and mode of use are described.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

II. THE CONDITIONS NECESSARY FOR THE PRODUCTION OF LOCAL HEAT IN THE LUNGS.

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It has been shown elsewhere (1) that the passage of diathermy currents of strengths equivalent to those used in therapy raises the deep temperature in those portions of the abdominal and thoracic viscera which lie between the electrodes only slightly (about $0.4^{\circ}\text{C}.$) above the rectal temperature. That the rectal temperature can be elevated in both anesthetized and unanesthetized animals was also shown. In one unpublished experiment (D 27) the rectal temperature of an anesthetized dog as recorded by a thermocouple was raised from $37.71^{\circ}\text{C}.$ to $40.48^{\circ}\text{C}.$ during the passage of a current of 2000 milliamperes for 45 minutes between electrodes placed laterally on the thorax. The current density here was 166 milliamperes per square inch of electrode surface, which exceeds that commonly employed for therapeutic purposes, and yet at the end of the period the lung temperature in four different parts of the lungs, as recorded by four thermocouples, was $40.83^{\circ}\text{C}.$, $40.85^{\circ}\text{C}.$, $40.85^{\circ}\text{C}.$, and $40.85^{\circ}\text{C}.$, or $0.37^{\circ}\text{C}.$ above the rectal temperature. A measurement of rectal temperature by a mercury thermometer made at the same time agreed exactly with the rectal thermocouple reading.

Furthermore, we have shown that sudden death permitted an abrupt rise of local temperature in the deep tissues between the electrodes without a corresponding rise in rectal temperature. This was observed in both intraabdominal and intrathoracic tissues, a fact which, together with the tendency of the lung temperature to approximate that of the rectum, suggested that the cooling of the lung was primarily a circulatory phenomenon rather than one due to the passage of air in and out of the lung.

To put this hypothesis to the test experiments were devised which had for their purpose a study of those conditions which would permit local heating of the lung during life.

EXPERIMENTAL.

In the part to follow experiments will be described in which the access of air or blood or both to one lung was prevented by various operative procedures. Temperature measurements were made in the altered lung with simultaneous records of temperature in the opposite lung, as well as in other parts of the body. It was discovered, as was anticipated, that the circulating blood, rather than the access of air, played the chief rôle in carrying away heat produced by diathermy.

Experiment D 14. Effect of Diathermy on Lung Temperature during (a) Blocking of the Left Bronchus, (b) Occluding the Left Branch of the Pulmonary Artery.—A male mongrel weighing 9.7 kilos was anesthetized by the intravenous injection of 2.91 gm. barbital-sodium dissolved in physiological saline. An additional 8 cc. of a 5 per cent solution of the drug was required to obtain complete anesthesia and relaxation. Tracheotomy was then performed. With the dog lying on his right side a thoracotomy was done through the fifth left interspace. A brass tube with flanged end was passed down the trachea and securely tied into the left bronchus just beyond the tracheal bifurcation. A similar, but shorter, brass tube with an equal bore, 7 mm., was inserted into the trachea parallel to the first one. A firm ligature was placed about the cut trachea so that both tubes were held firmly in place, and no air leaked about them.

By this procedure the air was partitioned, that passing down the one tube entering the left lung, that passing down the other entering the right lung. The access of air to either lung could therefore be prevented¹ by closing the mouth of the tube. To interrupt the blood flow to the lung a pneumatic cuff of the kind recently described by Moore and Binger (2) was placed about the left branch of the pulmonary artery and a tube communicating with the cuff brought out through the chest wall. By blowing air through this tube the cuff was inflated, thus effectively occluding the artery.

Lead-tin electrodes were applied laterally to the chest walls, care being taken to have the surfaces as parallel as possible. Several layers of gauze soaked in glycerol-salt mixture were interposed between the electrodes and the shaved skin to assure perfect contact. Thermocouple needles were then inserted into

¹ We wish to thank Dr. Richmond L. Moore, of this laboratory, for providing us with this technique.

the dog's lungs in the manner previously described (1). At the close of the experiment it was shown that two of the thermocouples lay in the left lower lobe, one in the right lower lobe, and one in contact with the right visceral pleura, probably in the interlobar space. A mercury thermometer was used to record rectal temperature.

After control readings of all temperatures had been recorded the current was turned on and its strength increased until the ammeter read 1200 milliamperes. After 40 minutes flow the rectal temperature rose by 1.35°C ., the temperature in the right lung by 1.18°C ., and the temperature in the left lung by 1.31°C .. The access of air to the left lung was then prevented by blocking the appropriate tube. No change in rate of heating of the left lung resulted from this procedure. In the succeeding half-hour the rectal temperature rose by 1.21°C ., the right lung by 0.95°C ., and the left lung, whose bronchus was now blocked, by 1.02°C ..

By opening the end of the tube the bronchial obstruction was released and air again permitted to enter the left lung. 11 minutes later the rubber cuff surrounding the left branch of the pulmonary artery was inflated, thereby shutting off part of the blood supply to the left lung. 5 minutes before interrupting the circulation in the left lung the dog's rectal temperature was 38.47°C ., 5 minutes after, 38.88°C .. The corresponding lung temperatures were—right, 38.63 – 38.85°C ., left 38.73 – 40.80°C .. In other words, whereas the temperature of the right lung rose 0.2°C . in this interval the temperature of the left lung suddenly rose 2.0°C . as the result of clamping the left branch of the pulmonary artery. The subsequent rate of heating of the two lungs was the same.

Later in the experiment the left bronchus was again occluded with no apparent effect on the rate of heating of the left lung. The animal was then suddenly killed by the intravenous injection of 20 cc. of chloroform. There resulted an abrupt and equal rise in the heat production in both lungs, the rectal temperature in the meanwhile falling. The temperature curves are shown in Fig. 1.

This experiment (D 14) is presented in some detail because it contains in it all the facts which are to be analysed in the remaining parts of this paper. The general conclusions suggested by it are these: (1) Prevention of access of air to one lung does not alter its rate of heating by diathermy, nor does it permit local heating in excess of the general systemic heating. (2) Interruption of the circulation through a branch of the pulmonary artery does, however, result in a sudden rise of temperature in the lung supplied by this branch. After the sudden initial rise heating continues at the original rate which in general is the rate of heating of the other lung and of the rectum. (3) Sudden death of the animal during diathermy results in a precipitous and equal increase in the temperature of both

lungs, the one with its artery occluded as well as the normal lung, and in a gradual fall in rectal temperature.

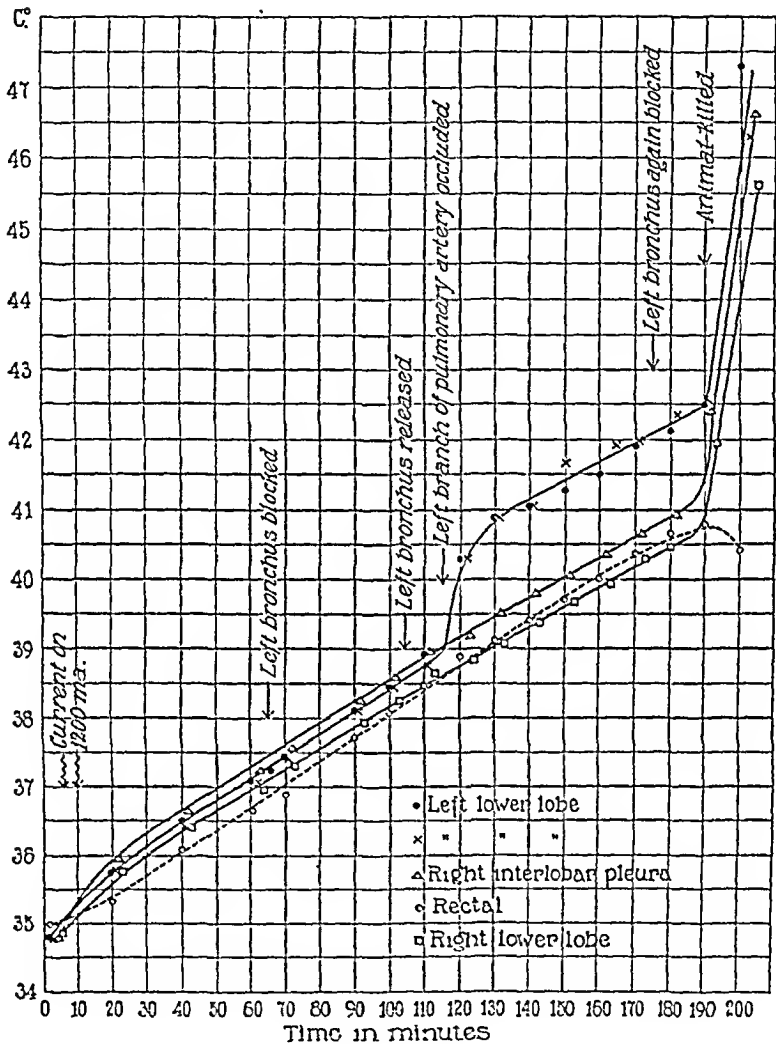


FIG. 1. Curve showing effect of (a) obstructing left bronchus, (b) occluding left branch of pulmonary artery, on heat developed in left lung as compared with heat developed in the right lung and rectum.

A further analysis of these various points follows:

Experiment D 21. Effect of Bronchus Blocking on Heat Production in the Lung.
—A female collie weighing 9.7 kilos, anesthetized with barbitol-sodium given

intravenously, was operated upon for the purpose of intubating the left bronchus. A diathermy current of 1200 milliamperes was passed between electrodes placed laterally on the chest. Temperature readings were made on thermocouples in the right and left lungs. During the current flow the left bronchus was hlocked and subsequently released. The temperature in the right lung was $39.74^{\circ}\text{C}.$, and in the left, before blocking the bronchus, was $39.75^{\circ}\text{C}.$ 13 minutes after hlocking the access of air to the left lung, it had warmed $0.94^{\circ}\text{C}.$, the right lung $0.83^{\circ}\text{C}.$ The maximum increase in temperature of the left lung over the right during the period of blocking was $0.38^{\circ}\text{C}.$ Whether this is a real effect or due to experimental error, we do not know. It is probably a real one as the agreements in lung temperatures in this experiment, as well as in other ones, before hlocking, were closer than this. In any case it shows, as does the last experiment, that prevention of access of air to one lung during diathermy of the thorax does not provide conditions which make for any significant degree of local heating.

Experiment D 15. Repetition of Arterial Occlusion Experiment.—It has already been shown that clamping the artery to one lung during diathermy results in a sudden increment of local heat. A second experiment on an anesthetized dog was performed with a pneumatic cuff placed about the left branch of the pulmonary artery. This cuff was alternately inflated and deflated with immediate changes in heat production in the lung.

The experimental results are so clearly shown in Fig. 2 that it seems superfluous to describe them at greater length. Four things should be noticed in particular: First, the smoothness of the curves; secondly, the fact that once the sudden rise has occurred there is a tendency for the lung to continue heating up at approximately the original rate; thirdly, that after clamping the artery to the left lung the temperature of the right lung falls below the rectal temperature to an extent not seen in experiments in which the pulmonary circulation is not disturbed. This is an interesting point which is also shown in Fig. 1. It probably signifies that on interrupting the circulation to the left lung there is an increased flow of blood through the right lung, with consequently more efficient cooling. The fourth point of interest shown in Fig. 2 has been mentioned before—it is the sudden heating up which occurs equally in the two lungs, upon the death of the animal.

An explanation for some of these facts is furnished by the next experiment, in which the root of one lung was ligated during diathermy. This was done for the purpose of interrupting the circulation through the bronchial artery, as well as through the pulmonary artery. It was

thought that in all probability the blood flow through the bronchial vessels would be sufficient to maintain some degree of cooling even

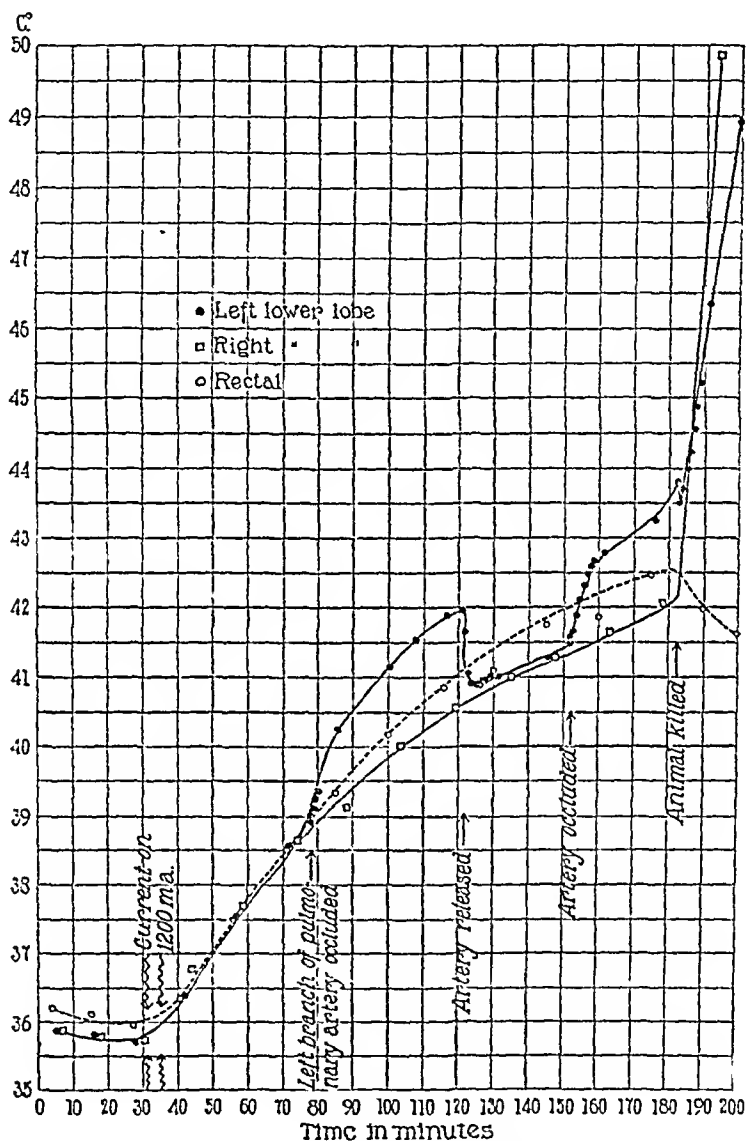


FIG. 2. Curve showing comparison of heat developed in right and left lungs and rectum, with effect of clamping and releasing artery to the left lung.

after the circulation through the pulmonary artery had been shut off, and thus prevent the temperature in the lung from rising precipitously as it does at death.

Experiment D 17. Ligation of Root of Right Lung during Diathermy.—A female fox-terrier under barbital anesthesia was prepared for the experiment by a right sided thoracotomy in which a loose ligature was placed around the root of the right lung. The ligature ends were brought out through the deep muscles of the back in such a manner that subsequent traction and ligation of the ends would completely shut off the circulation to the right lung. After repair of the chest wall and application of the electrodes, thermocouples were inserted into both lungs. At the close of the experiment the position of the thermocouples was confirmed. The animal was put on artificial respiration by the intratracheal insufflation of an interrupted stream of air. A current of 1200 milliamperes was then allowed to pass between the electrodes. In the half-hour previous to vessel ligation the right lung temperature rose from 36.12°C. to 40.04°C. These figures represent mean readings of two thermocouples in the right lower lobe. Simultaneously the temperature in the left lower lobe had risen from 36.21°C. to 40.32°C., showing that the two lungs were heating at approximately the same rate. The change in rectal temperature was of a similar grade.

The ligature about the right hilum was then suddenly tightened, with the result that in the next 6 minutes the right lung temperature increased by more than 2°C., the left by 0.6°C. 15 minutes later the right was over 3.5°C. warmer than the left. After killing the animal the rate of temperature increase in the right lung remained relatively constant, while the temperature in the opposite lung suddenly mounted precipitously. The changes are graphically shown in Fig. 3.

In another and similar experiment (D 19) the left lung root vessels of a dog were ligated before diathermy, leaving the bronchus, however, free. A current, again of 1200 milliamperes, was passed through the dog's thorax, and temperature measurements were made. In this experiment, as will be seen in Fig. 4, the right lung temperature increased at the same general rate as the rectal temperature; the left lung, on the other hand, at a much faster rate. Subsequent ligation of the left bronchus had little influence on the rate of warming of the left lung, whose root vessels had previously been tied. The dog was killed by the intravenous injection of 35 cc. of chloroform. This resulted in a precipitous rise in temperature in the unligated lung similar to that which had occurred in the ligated lung when the current was first turned on.

These last two experiments, we believe, indicate that the bronchial circulation may play an important part in carrying away the heat produced in the lung by the passage of high frequency currents. The blood flowing through the bronchial artery normally drains into the pulmonary veins. By ligating these structures at the lung root the whole circulation to the lung can be stopped, which cannot be accomplished by ligation of the main branch of the pulmonary artery alone. When the circulation to the lung is thus interrupted (even though its

bronchus remains patent) it is heated rapidly by the diathermy current to a point several degrees above the temperature prevailing in the

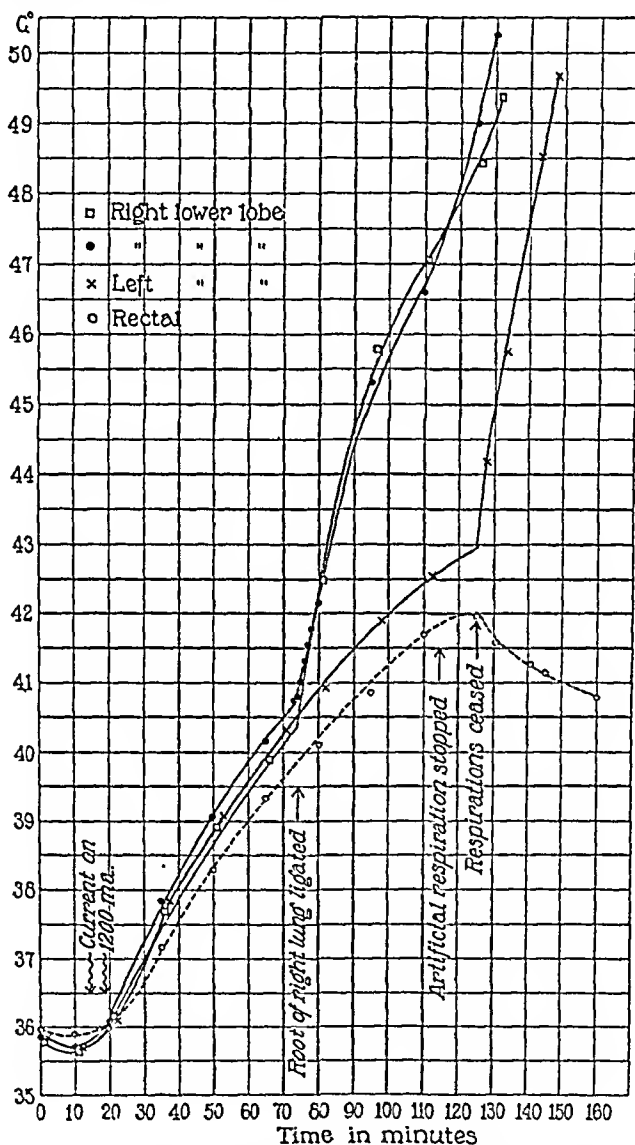


FIG. 3. Curve showing comparison of heat developed in right and left lungs and rectum. Effect of ligating the root of the right lung, and effect of sudden death.

normal lung or in the rectum, and at a rate equivalent to the rate of heating which occurs in the normal lung after death.

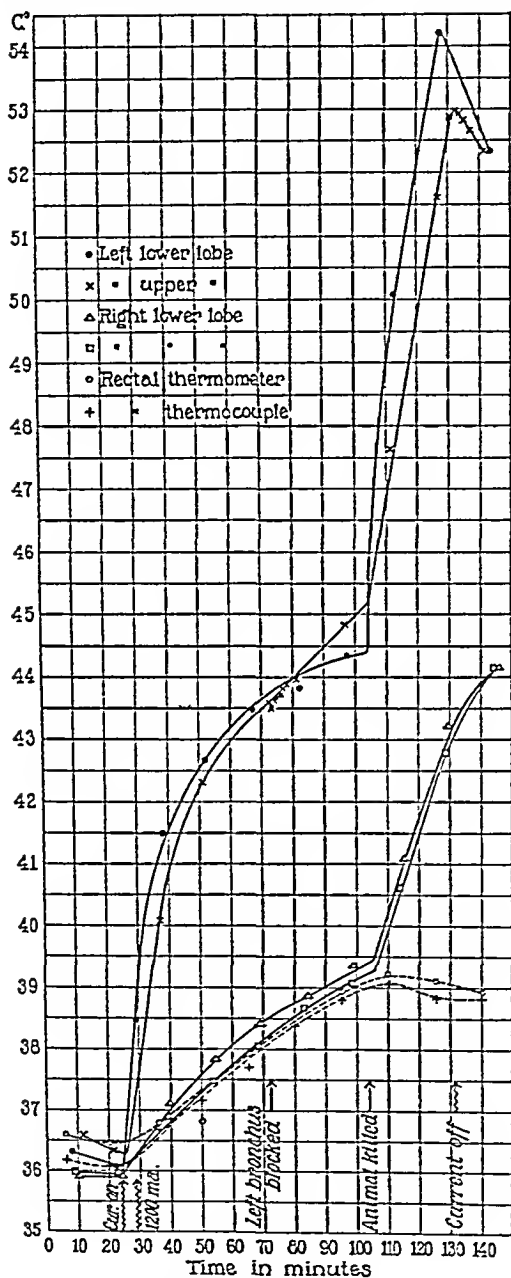


FIG. 4. Curve showing relative rates of heating of normal lung and lung whose root vessels have been ligated.

DISCUSSION.

Under the circumstances of these experiments, at least, it seems apparent that blood cooling is a more effective mechanism in removing heat from the lung than air cooling. Indeed, it is probable that the condition most needed for the production of local heat in the lung, by the passage of the diathermy current, is a partially or completely obstructed circulation in the pulmonary and bronchial vessels. The bearing of this on the problem of local heat production in the pneumonic lung is obvious, and will be experimentally dealt with later.

SUMMARY AND CONCLUSIONS.

1. Prevention of the access of air to one lung, while its circulation is intact, results in little, if any, change in the rate of heating of the lung by the diathermy current.

2. Occlusion of a main branch of the pulmonary artery during the flow of the current results in a sudden rise in temperature in the lung whose artery has been occluded, with subsequent heating, however, at the original rate. Under these circumstances death of the animal is accompanied by a precipitous rise in the temperature of both lungs.

3. When the pulmonary veins as well as the artery to one lung are ligated the circulation through the bronchial vessels is also stopped. Diathermy then results in a local rise in temperature in the lung equivalent to that seen in the other lung after death.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

III. THE TEMPERATURE OF THE CIRCULATING BLOOD.

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We have convinced ourselves by experiment that the normal lung can be heated only slightly above the systemic temperature by the application of high frequency currents to a dog's thorax (1). Interference with the circulation of blood to the lung, however, either by clamping one of the main branches of the pulmonary artery, or by ligation of all the root vessels, provides the conditions necessary for local deep heating (2). The implication that the circulating blood serves to carry away the heat produced in the lung seems obvious. Such an interpretation is in harmony with the physiology of heat distribution and temperature regulation (3). It is probable that together with its many other equilibrating functions the blood is a fairly ideal medium for distribution and maintenance of a uniform temperature.

Methods.

The methods which we have previously used for deep temperature measurements were easily modified for the purpose of recording intravascular temperatures. In place of the thermocouple needle we used similar copper-constantan couples housed in No. 14 gauge rolled steel tubing 10 cm. long. The junction was soldered to the end of the tubing, the solder forming a smooth, blunt tip. Capillary rubber tubing with a 3 mm. outside diameter was used to protect the wires. This was sufficiently small to permit its passage through the femoral vein or artery into the vena cava and aorta, or through the jugular and carotid, into the right and left heart. The steel and rubber tubing were smeared with a petroleum jelly of high melting point to inhibit blood clotting about them.

The methods for applying diathermy were those previously described.

EXPERIMENTAL.

The Temperature of Arterial and Venous Blood.

Experiment D 24.—A male mongrel hound weighing 21.7 kilos was anesthetized by the intravenous injection of barbital-sodium. The right femoral artery and left femoral vein were exposed and opened sufficiently to admit the passage of two thermocouples into their lumina. These thermocouples were then pushed up into aorta and vena cava. At the conclusion of the experiment the aortic thermocouple was found in the abdominal aorta just under the diaphragm; the other in the vena cava at the opening of the superior hepatic vein. The dog's rectal temperature was recorded by a mercury thermometer calibrated in $0.10^{\circ}\text{C}.$, which could be read accurately to about $0.02^{\circ}\text{C}.$ A comparison of rectal thermometer readings and temperatures in the right femoral artery and aorta showed an average agreement within $0.02^{\circ}\text{C}.$ in five readings taken at approximately 5 minute intervals for $\frac{1}{2}$ hour. This agreement held even though the thermocouple position was altered and advanced up the artery from a point 10 cm. above its entrance into the vessel to a point 30 cm. above its entrance into the vessel. The latter position was the one found at autopsy, namely, in the abdominal aorta immediately under the diaphragm. It should be stated that when the animal began to lose heat and the rectal temperature fell, the agreement was not so close as that just mentioned.

Measurements of venous blood temperatures made simultaneously showed a decided difference from both arterial and rectal temperatures. At the time of the first three observations the venous temperature was $0.25\text{--}0.33^{\circ}\text{C}.$ below the arterial. With the further advance of the thermocouple upward into the vena cava this difference became less until a point was reached 30 cm. above the entrance of the thermocouple into the vein, which corresponded to the level of the hepatic veins, where the venous temperature was higher than both arterial and rectal temperatures by slightly more than $0.10^{\circ}\text{C}.$

In the chambers of the heart the normal relationship, in the anesthetized dog at least, is for the temperature in the left ventricle to be slightly below the temperature in the right auricle. This difference amounts to less than $0.2^{\circ}\text{C}.$ and usually more than $0.05^{\circ}\text{C}.$, and may be supposed to be due to heat loss in the blood as it traverses the lungs. The difference between temperature readings obtained by passing thermocouples down the right jugular vein and left carotid artery into the heart in three dogs is shown in Table I. As will be understood from what has just been stated, the venous temperature will depend not only on the location of the thermocouple but on the relative amount of blood from the abdominal viscera flowing into the right auricle.

In another experiment (D 40) in which great care was taken to prevent cooling of the animal, the rectal temperature rose by nearly 0.1°C . during the period of observation. In this dog the temperature of the blood was lower than that registered by a thermocouple in the rectum. The venous and arterial blood temperature, however, exhibited the relationships just described. As the venous thermocouple was advanced along the femoral vein and up into the vena cava the recorded temperature continued to mount until it exceeded the arterial blood temperature by 0.04°C . Both intravascular thermocouples were then occupying positions 47.5 cm. above the points of entrance in the femoral vessels, one of them lying in the vena cava, the other in the aorta at a level half-way between the dome of the diaphragm and the heart. These relative temperatures are

TABLE I.

Comparison of Temperatures Recorded by Passing Thermocouples down Right Jugular Vein and Left Carotid Artery.

Experiment No.	Reading on thermocouple passed down right jugular vein	Reading on thermocouple passed down left carotid artery	Difference
	$^{\circ}\text{C}$.	$^{\circ}\text{C}$.	$^{\circ}\text{C}$.
D 25	36.37	36.29	-0.08
	36.35	36.33	-0.02
D 26	34.91	34.87	-0.04
	34.81	34.75	-0.06
	34.75	34.69	-0.06
D 29	37.07	36.88	-0.19
	37.06	36.87	-0.19
	37.11	36.93	-0.18

graphically shown in Fig. 1. The upward inclination of the temperature curve for the arterial thermocouple was the result of the gradual rise in body temperature as illustrated by the similar curve for the rectal temperature. It should not be confused with the striking rise in venous temperature which occurs as the thermocouple is advanced upward into the vena cava.

The Temperature of the Arterial and Venous Blood during Diathermy.

The passage of the diathermy current between electrodes placed laterally on the dog's thorax results not only in an elevation of rectal temperature and lung temperature, as has been shown, but in an equivalent rise in the temperature of the circulating blood. The

arterial blood in the left heart or aorta, normally slightly cooler than the venous blood, rises to a greater degree than does the venous. The usual relationship is, therefore, reversed. For example, in Experiment D 25 the temperature in the left ventricle before diathermy was $36.29^{\circ}\text{C}.$, in the right auricle, $36.37^{\circ}\text{C}.$ After the onset of current flow this relationship was reversed, the temperature in the left heart now exceeding that in the right by $0.14\text{--}0.28^{\circ}\text{C}.$ The difference, though not great, persisted as long as the current flow continued and disappeared as soon as the current was shut off, the

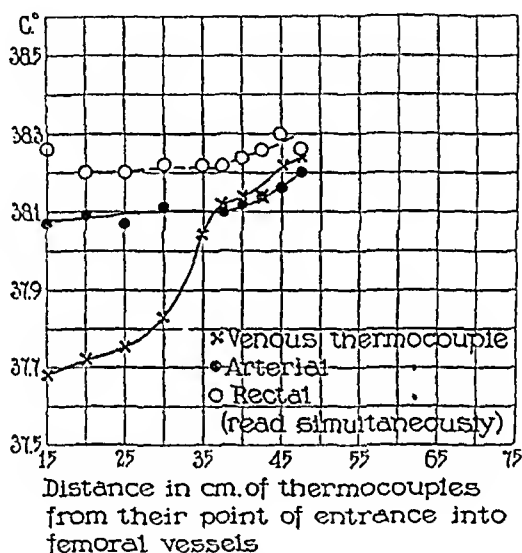


FIG. 1. Curve showing temperatures at various levels in aorta and vena cava with simultaneous rectal temperatures.

venous being now $0.07^{\circ}\text{C}.$ above arterial. In another experiment (D 26) the left carotid blood was $0.06^{\circ}\text{C}.$ cooler than the right jugular before the passage of 1500 milliamperes between the electrodes. After $\frac{1}{2}$ hour's diathermy, the arterial blood temperature exceeded the venous by $0.2^{\circ}\text{C}.$

Changes of a similar order of magnitude appeared in Experiment D 40. These are graphically plotted in Fig. 2, and in Table II will be found a comparison of the temperatures of arterial and venous blood before and during the passage of the diathermy current in four separate experiments.

TABLE II.

Comparison of Arterial and Venous Blood Temperatures before and during Diathermy.

Experiment No.	Temperature before diathermy			Temperature during diathermy		
	Arterial	Venous	Difference	Arterial	Venous	Difference
	°C.	°C.	°C.	°C.	°C.	°C.
D 25	36.29	36.37	-0.08	39.70	39.47	+0.23
D 26	34.69	34.75	-0.06	41.83	41.73	+0.10
D 29	36.88	37.06	-0.18	38.95	38.99	+0.04
D 40	38.22	38.29	-0.07	40.01	39.89	+0.12

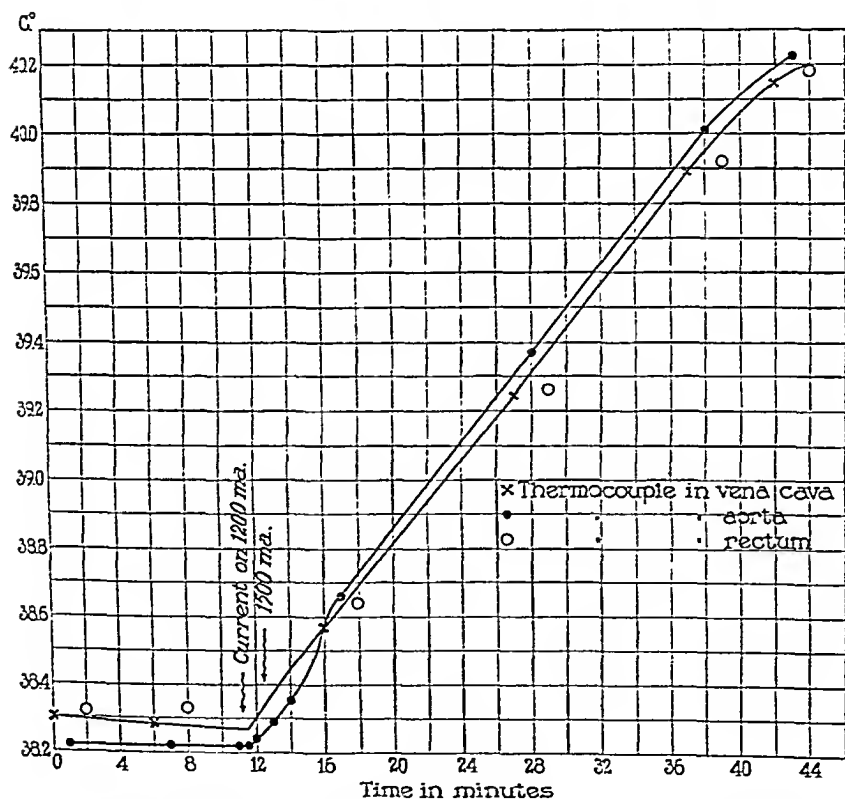


FIG. 2. Curve showing temperature of right and left heart blood before and during diathermy.

DISCUSSION.

The facts presented in this paper may be regarded as further evidence of the effective cooling of the lung during diathermy by the blood circulating through it. It can be estimated roughly, assuming a minute volume of blood flow through the lungs of 2.50 liters, and a rise of $0.2^{\circ}\text{C}.$ in arterial blood temperature above venous, that approximately half of a large calorie of heat is being removed from the lungs per minute. This is evidently sufficient to prevent any marked degree of local heating.

SUMMARY AND CONCLUSION.

1. A method of measuring intravascular temperatures in anesthetized dogs has been described.
2. The temperature in the abdominal aorta is uniform throughout, and varies only with the systemic temperature.
3. The temperature in the inferior vena cava rises as the thermocouple approaches the heart, reaching its maximum at about the level of the hepatic veins. Between the hepatic veins and the right chambers of the heart there is no further elevation in venous temperature.
4. The temperature of the right heart blood normally exceeds that of the left heart blood by $0.05\text{--}0.2^{\circ}\text{C}.$
5. During the application of high frequency currents to the thorax, this relationship is reversed.
6. This indicates that the lungs are being heated but that the blood passing through the pulmonary vessels is removing the heat at approximately the rate of production.

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THE SOLUBLE SPECIFIC SUBSTANCE OF FRIEDLÄNDER'S BACILLUS.

III. ON THE ISOLATION AND PROPERTIES OF THE SPECIFIC CARBOHYDRATES FROM TYPES A AND C FRIEDLÄNDER BACILLUS.

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The parallelism between capsular synthesis and virulence among pathogenic bacteria is one which has been extensively investigated, but researches concerning the chemical nature of encapsulating substances have indeed been very few. Fürst (1) has described the encapsulating substance of the Friedländer bacillus. He believed it to be a mucoprotein-like substance. Hamm (2) contended that the capsule substance contained nucleoprotein. Preisz (3) has attempted unsuccessfully to immunize animals with the capsular substance from the anthrax bacillus. More recently Toennissen (4) succeeded in isolating a nitrogen-free polysaccharide from the Friedländer bacillus, which he believed to be identical with the capsular material of this microorganism. Similar polysaccharides have been isolated from cultures of the bacilli of the *aerogenes* group (5), from *Streptococcus hornensis* (6), and even from certain yeasts (7).

Strange as it may seem, none of these earlier investigators ascribed immunological significance to these bacterial carbohydrates. The so called soluble specific substance of the pneumococcus, first observed by Dochez and Avery (8), has been identified with the polysaccharide portion of the organism. An extensive investigation into the chemical and immunological nature of carbohydrates from the pneumococcus group has been carried out in this laboratory (9). Similar investigations have recently been extended to the group of Friedländer bacilli.

From a strain of Friedländer's bacillus, Mueller, Smith, and Litarczek (10) have recorded the isolation of carbohydrate-containing material which at high dilutions reacted specifically with homologous antibacterial serum. It has been shown by Julianelle (11)

that bacilli of this group are separable immunologically into sharply defined specific types. These types have been designated as Type A, Type B, and Type C; the remaining unclassified strains were placed in a heterologous group X. The method of isolation of the specific carbohydrate of Type B Friedländer bacillus has been given in detail (12). The present communication, which concludes a systematic description of the specific polysaccharides from the three fixed types of Friedländer's bacillus deals with a description of the polysaccharides from the Type A and Type C microorganisms. A more detailed study concerning the nature of the hydrolytic products of the Type A specific carbohydrate is reported separately (13).

EXPERIMENTAL.

I.

1. Isolation of the Specific Carbohydrate of Type A Friedländer Bacillus.

Autoclaved washings from 50 large Blake bottles of 72 hour cultures of Type A Friedländer bacillus, grown on solid agar containing 0.5 per cent dextrose at pH 7.6, were treated with a sterile solution of purified trypsin.¹ After standing overnight at 37° the mucoid-like washings (containing the specific carbohydrate) became nearly clear. The faintly alkaline solution was treated with 50 gm. of sodium acetate and 2 volumes of alcohol. A flocculent precipitate, containing all the specific carbohydrate in an impure state, settled out on standing. The supernatant liquid which gave no test for specific substance with antiserum, was discarded and the precipitate was separated by centrifugation. After dissolving the precipitate in a liter of water an opalescent solution was secured. By adding acetic acid it was found that a small amount of non-specific nitrogenous material could be separated by centrifugation. The solution of impure specific carbohydrate thus obtained was again treated with 2 volumes of alcohol and the resulting precipitate of specific carbohydrate was again separated by centrifugation. This process of alcoholic precipitation (a process which was found to eliminate nitrogenous impurities) was repeated five or six times until finally a snow-white product was secured, which gave a perfectly clear solution when dissolved in water. This solution, now at a volume of 500 cc., was treated with 50 cc. of 1:1 hydrochloric acid and was dialysed in collodion bags against successive changes of distilled water

¹ 8 gm. of commercial trypsin were dissolved in 200 cc. of water, acidified with N/1 hydrochloric acid until Congo red paper turned a faint purple. The solution was centrifuged, and the supernatant liquid was neutralized with N/1 sodium hydroxide, and again centrifuged.

until free from Cl ion both within and without the bag. The dialysed solution of the carbohydrate was concentrated to 200 cc. *in vacuo*, and was then treated with 400 cc. of redistilled glacial acetic acid. After standing 2 or 3 hours a flocculent precipitate settled out of the solution. This non-specific precipitate was separated by centrifugation and was discarded. The clear supernatant liquid, which contained all the specific material, was concentrated to small volume *in vacuo*, and was then poured into 10 volumes of redistilled acetone containing a trace of hydrochloric acid. Between 5 and 6 gm. of polysaccharide were recovered.

2. Properties of the Soluble Specific Substance of Type A Friedländer Bacillus.

The soluble specific substance of the strain of Type A Friedländer's bacillus is a white amorphous powder with marked acidic properties,

TABLE I.

Soluble Specific Substance of the Friedländer Bacillus Type A.

Preparation No.	$[\alpha]_D$	Acid equivalent	Ash	C	H	N	Reducing sugars on hydrolysis (as glucose)	Highest dilution giving precipitate with immune rabbit serum
							per cent	
117-A	-103.4°	430	0.0	43.98	6.00	0.1	64	1:2,000,000
118	-101°	430	0.0			0.0	65	1:2,000,000
120	-105°	445	0.0			0.0	68	1:2,000,000
122	-100°	435	0.0			0.1	65	1,2:000,000
123	-101°	430	0.0			0.0	67	1,2:000,000

the acid equivalent being approximately 430. A 1:200 solution turns Congo red paper blue. Such solutions are not precipitated by copper or silver ions. The polysaccharide is incompletely precipitated by solutions of phosphotungstic acid, barium hydroxide, and uranyl nitrate, but is completely precipitated by both neutral and basic lead acetate solutions. The nitrogen-free product, when oxidized with sodium peroxide, gives no test for sulfur or phosphorus. The polysaccharide gives no color test with potassium iodide-iodine solution. Acid hydrolysis of the material gives a reducing solution which shows a strong naphthoresorcinol test. Attempts were made to purify the specific substance by partial precipitation with barium hydroxide, uranyl nitrate, and by adsorption on alumina, but in each

instance a substance with properties similar to the original material was recovered. The properties of the various preparations were remarkably uniform as shown by Table I.

II.

1. Isolation of the Type C Soluble Specific Substance of Friedländer's Bacillus.

The specific carbohydrate of the strain of Type C Friedländer's bacillus was obtained from autoclaved washings of cultures grown on dextrose agar media exactly as was the carbohydrate from Type A. The product was isolated in an ash-free form by the addition of 2 volumes of alcohol to a 1:50 solution of carbohydrate, containing 10 cc. of 1:1 hydrochloric acid. After standing at 0° for 1 hour the polysaccharide separated as a flocculent precipitate which was filtered on a hardened paper and washed free from chloride with absolute alcohol. The yield from 100 Blake bottles was about 3 gm.

2. Properties of the Soluble Specific Substance of Type C Friedländer Bacillus.

The material thus isolated was an amorphous water-soluble powder, free from nitrogenous impurities and ash. The substance is a strong acid, for it turns moist Congo red paper blue. A 1:200 solution is not precipitated by solutions of silver nitrate, copper sulfate, phosphotungstic acid, or ammonium molybdate, but is precipitated by solutions of uranyl nitrate, neutral and basic lead acetates, and by concentrated solutions of barium hydroxide. The carbohydrate gives no color with iodine-potassium iodide solution. The substance has a specific rotation of +100° and its acid equivalent is approximately 680. It reacts with immune rabbit serum at a dilution of 1:2 million.

The properties of various preparations are shown in Table II.

3. Hydrolysis of the Type C Specific Substance.

1.5 gm. of Preparation 3-A were boiled for 5 hours with 50 cc. of N/1 sulfuric acid under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide, the solution was boiled with an excess of calcium carbonate and a small amount of norit, and was filtered. The clear filtrate was evaporated to dryness *in vacuo*, and the residue was extracted with methyl alcohol, the alcoholic solution was filtered and was then evaporated to dryness *in vacuo*. (In this manner the free sugars were separated from the sugar acids, which were

present as their calcium salts and which were insoluble in methyl alcohol.) The residue of free sugars was dissolved in water and diluted to 100 cc. in a volumetric flask. An analysis by the Shaffer-Hartmann method showed 0.84 gm. of reducing sugars calculated as glucose. On the basis of this weight the solution showed a specific optical rotation of $+48.2^\circ$.

TABLE II.

Soluble Specific Substance of the Friedländer Bacillus Type C.

Preparation No.	$[\alpha]_D$	Acid equivalent	Ash	N	Reducing sugars on hydrolysis (as glucose)	Highest dilution giving a precipitate with immune rabbit serum
					per cent	
1	$+90.0^\circ$	610	0.0	0.5	75.0	1:2,000,000
1-A	$+100.0^\circ$	680	0.0	0.0	73.1	1:2,000,000
2	$+100.0^\circ$	681	0.0	0.0	—	1:2,000,000
3	$+85.0^\circ$	575	0.0	0.0	—	1:2,000,000
3-A	$+101.0^\circ$	674	0.0	0.0	75.0	1:2,000,000
3-B	$+58.0^\circ$	794	0.0	0.3	55.0	1:1,000,000

Preparation 1 was the first preparation isolated and had a rather high nitrogen content. The nitrogen was removed (Preparation 1-A) by precipitating the substance in a 1:100 solution with barium hydroxide saturated at 60° and then recovering the polysaccharide from the precipitate, by treating it with an excess of sulfuric acid, centrifuging off the barium sulfate, and reprecipitating the polysaccharide, in the presence of acid, with alcohol.

Preparation 3 was obviously impure. It was separated into two fractions by treating a 1:100 solution of the carbohydrate with a slight excess of barium hydroxide saturated at 50° . The precipitate was centrifuged off and the carbohydrate was recovered in the usual way (Preparation 3-A). The supernatant liquid from this barium hydroxide precipitation still contained specific material. This was removed by precipitation with alcohol. The precipitate, Preparation 3-B, was obviously an impure substance still containing specific material. Attempts at further purification of the preparations by adsorption on alumina, precipitation with uranyl nitrate, etc., yielded products with properties identical with the starting material.

Half of the solution was treated with 3.5 mols of phenylhydrazine acetate and an osazone was isolated in the usual manner. 0.1 gm. was recovered. The substance melted at $201-203^\circ$ and had an $[\alpha]_D = -54.3^\circ$ mutarotating to -20.0° after 48 hours.

The second half of the solution was evaporated to 1 cc. and was oxidized with

3 cc. of 1:1 nitric acid. 0.070 gm. of potassium acid saccharate was obtained which was recrystallized from 1 cc. of water. 0.030 gm. was recovered.

0.005326 gm. substance gave 0.001853 gm. K_2SO_4 .

Calculated for $COOH(CHOH)_4COOK$, K 15.75 per cent.

Found

K 15.58 per cent.

It is evident that this sugar, which represents the greater part of the hydrolytic products of this soluble specific substance, is glucose. 0.5 gm. of residue was left from the methyl alcoholic extract of the original total hydrolysate. This material appeared to be the calcium salt of a sugar acid. It was an impure substance, judging from its color. It gave a strong naphthoresorcinol test and a strong reduction test. It is possibly the salt of an aldobionic acid such as has been found among the hydrolysis products of other specifically reacting polysaccharides. The substance will be investigated further when more material is available.

DISCUSSION.

It is clear from the foregoing experiments that strains of Friedländer's bacillus of Types A and C yield, on fractionation, two chemically distinct nitrogen-free polysaccharides with highly specific properties. Both are strong acids and both contain glucuronic acid, or an isomer, within their molecules as shown by the naphthoresorcinol test. The polysaccharides themselves are non-reducing, but on hydrolysis with mineral acids they yield reducing sugars. In both instances, as in the case of the pneumococci, specific function and carbohydrate are apparently inseparable.

On comparing the specific carbohydrates from Type B and Type C Friedländer bacillus, an unusual similarity in properties is to be observed. These substances, however, possess two distinct differences. Immunologically they show no cross-relationship. Their solubility in water in the pure state and their whole behavior during purification are entirely different. In pure form the Type B substance is difficultly soluble in water whereas the Type C substance is readily soluble. The Type B carbohydrate may be readily precipitated by alcohol in the presence of hydrochloric acid. The Type C substance, on the other hand, precipitates completely only after standing at 0° for an hour or more. The fact that two substances

so alike in physical properties are totally dissimilar in immunological reactions, may possibly be explained on the basis of slight differences in the intramolecular linkages of sugar to sugar, or of sugar to sugar acid.

SUMMARY.

1. Methods are given for the isolation of specifically reacting nitrogen-free polysaccharides from Type A and Type C Friedländer's bacillus.

2. The properties of these specific carbohydrates have been outlined.

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THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

IV. THE ACTION OF DIPHTHERIA TOXIN IN SPLENECTOMIZED AND BLOCKED MICE.

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While the relation of the reticulo-endothelial system to the production of an artificially acquired immunity has been studied in the past from various angles, it is only recently that attempts have been made to bring phenomena of natural immunity into connection with the same system of cells. Our knowledge of the mechanism of natural resistance in certain animals to certain infections and intoxications has so far been limited almost entirely to the observation that such insusceptibility, with a few exceptions, is not due to humoral immune bodies and hence is not transmissible in most cases. In some instances, well defined physiological properties, such as a higher or a lower body temperature, can be held responsible for this phenomenon. Ehrlich did not actually advance our knowledge on this subject by attempting to explain the phenomenon on the basis of lack of suitable receptors in the tissue of the respective animals. In 1914, Lewis and Margot (1) published experiments which seemed to show that removal of the spleen from albino mice greatly increased their resistance to infection with *B. tuberculosis*. Later, Murphy and Ellis (2), however, were able to explain the anomalous results of the above mentioned authors. They found that mice splenectomized a short time prior to inoculation with *B. tuberculosis* were distinctly more susceptible than normal animals, while, with the increase of the interval between splenectomy and infection, the mice gradually showed a greater resistance than the controls. This increased resistance they explained by a secondary

hypertrophy of the remaining lymphoid tissue. Kyes (3) first definitely connected natural resistance with the fixed phagocytic cells of the endothelial system by his observations on the rôle of the hemophages of the liver and spleen in the natural immunity of the pigeon to pneumococcus infection. In 1925 Singer (4) reported on a breach of natural immunity effected by blockade of the reticulo-endothelial system. He was able, after blocking the reticulo-endothelial system of hens by intravenous injections of India ink, successfully to infect the fowls with *B. anthracis*, while the non-blocked controls remained refractory. Jelin's (5) recent publications likewise emphasize the importance of the reticulo-endothelial system for the mechanism of natural immunity against saprophytic organisms and more particularly for the conditions which determine the course of typhoid fever infection and intoxication in rabbits.

The relative insusceptibility of rats and mice to diphtheria toxin is well known. In the case of mice, it was first observed by Loeffler (6) in 1884 and has since been confirmed by a great number of authors. Roux and Yersin (7) and Behring and Kitashima (8) have been able to kill mice only by employing extraordinarily large doses of diphtheria toxin, while Loeffler failed entirely to infect them. Glenny and Allen (9) found that, when administered intravenously, the M.F.D. of a particular diphtheria toxin for mice was 60 times the subcutaneous M.F.D. for the guinea pig, while 100 times this dose had to be given to kill mice by the intramuscular route. Kolle and Schlossberger (10) reinvestigated the question and concluded that mice were practically insusceptible to large doses of diphtheria toxin, but were generally susceptible to fairly small doses of living bacilli. These authors also found no antitoxin in the blood of mice as had previously been described for rats by a large number of authors (Aronson (11), Koudrevetzky (12), Kuprianow (13), Cobbett (14), Goodman (15), Petitt (16), Coca, Russel, and Baughman (17)). Hippke (18) has been able only partially to confirm the one contention of Kolle and Schlossberger since he found but six strains, out of fifteen, to be virulent for mice. Members of the British Medical Research Council (19) also repeated Kolle and Schlossberger's work, using the same and even larger doses of diphtheria bacilli without obtaining regular results. Wolff (20), on the other hand, corroborates Kolle and Schlossberger's statements regarding the susceptibility of mice to the infection with living bacilli, and furthermore reports that he has succeeded in killing mice by repeated intravenous or intramuscular injections of fairly large doses of diphtheria toxin.

From a review of literature it would appear that the mouse is, to a large extent, insusceptible to diphtheria infection and intoxication,

although this immunity is by no means complete. It has seemed of interest to investigate whether the natural immunity of the mouse to diphtheria toxin and diphtheria bacilli is dependent upon the presence of an intact reticulo-endothelial system, by comparing the action of a highly potent diphtheria toxin and of a highly virulent diphtheria strain in normal mice with their action in mice which have been (1) blocked by intravenous injections of India ink, (2) splenectomized,¹ (3) blocked and splenectomized.

EXPERIMENTAL WORK.

Diphtheria toxin with and without phenol was used as well as diphtheria toxin concentrated by ultrafiltration. For the preparation of the latter we are indebted to Mr. D. Roelkey of this laboratory. The toxin was given by the intraperitoneal and the intravenous route in different experiments. The mice ranged in weight between 24 and 30 gm. Inasmuch as the pathological lesions at autopsy were not as characteristic as seen in other species, cultures were made on a blood plate from the heart's blood and the peritoneal exudate of the dead animals in order to disclose any intercurrent infection.

Series I.—Phenolized diphtheria toxin, No. 411a, having a M.F.D. of 0.0025 cc. for the guinea pig, was employed in this series. It was given intraperitoneally in amounts of 400 M.F.D. (1 cc.), 300 M.F.D. (0.75 cc.), 200 M.F.D. (0.5 cc.), and 100 M.F.D. (0.25 cc.), the volume of the dose in each case being 1 cc. Four groups of mice received these doses of toxin, each group consisting of eight animals: two normal controls, two animals which had received an intravenous injection of India ink (1 cc. 1:15) 2 days previously, two animals which had been splenectomized the day before, and two animals in which the ink injection had been combined with splenectomy. The eight mice which were injected with the 400 M.F.D. died in from 25 to 51 hours, those injected with 300 M.F.D. were found dead after from 34 hours to about 10 days. It should be stated, however, that in the latter group culture from the animal dying after 34 hours revealed the presence of hemolytic streptococci in both the heart's blood and the peritoneal exudate. In the group which received 200 M.F.D. death occurred in from 8 to 17 days, while all the mice which had received 100 M.F.D. survived. There was no definite and uniform difference between the control and the experimental animals in the length of time they lived after injection of the various doses of toxin. Death occurred rather irregularly within each group, demonstrating the wide margin of individual varia-

¹The operations were performed by using ether as an anesthetic.

tion in susceptibility of mice to diphtheria toxin as compared with the uniform reaction of guinea pigs to this poison.

Series II.—Diphtheria toxin without phenol was used in this series in which the effect of intravenous injections was studied. Toxin without preservative was selected, because it was found in preliminary experiments that phenolized toxin (0.5 per cent) killed mice immediately in intravenous doses of 0.75 cc., and even doses of 0.5 cc. provoked an extremely violent, immediate reaction. Broth, without phenol, injected in similar amounts intravenously, caused only a very slight transitory peptone shock. This toxin, which had a M.F.D. of 0.002 cc. for the guinea pig, was given in a uniform volume of 1 cc. in amounts of 200 M.F.D. (0.4 cc.), 150 M.F.D. (0.3 cc.), 100 M.F.D. (0.2 cc.), and 75 M.F.D. (0.15 cc.) intravenously to four similar groups of mice arranged as in the first series. The eight mice which had received the 200 M.F.D. died in from $4\frac{1}{2}$ to $7\frac{1}{2}$ days, while death occurred in the second group which had received 150 M.F.D., in from approximately .5 to 9 days. With the mice injected with 100 M.F.D. death was prolonged until about 11 days, while in the last group which had received 75 M.F.D., only one normal mouse died after $9\frac{1}{2}$ days. Again, death occurred within each group rather irregularly among duplicate animals, and no significant difference between experimental and control animals became apparent in the length of time they lived.

Series III.—Finally, a last series of experiments was carried out with diphtheria toxin (411a) which had been concentrated by ultrafiltration from an original volume of from 60 cc. to 6 gm. The original M.F.D., for guinea pigs, of 0.0025 cc. was thus decreased, as determined by animal tests, to 0.00025 cc. By using this concentrated and refined product it was possible to inject larger amounts intravenously without fear of a reaction induced by the excess of protein and phenol. Doses of 600 M.F.D. (0.15 cc.), 400 M.F.D. (0.1 cc.), 200 M.F.D. (0.05 cc.), and 100 M.F.D. (0.025 cc.) were injected intravenously in a uniform volume of 0.5 cc. into thirty-two mice, arranged in four similar groups as described before. With the first group which had received 600 M.F.D., death occurred in from 20 to 88 hours, in the second group (400 M.F.D.), the animals lived from 29 hours to 6 days. 200 M.F.D. caused death in from $5\frac{1}{2}$ to $8\frac{1}{2}$ days, while in the last group (100 M.F.D.) one splenectomized and one normal mouse survived; the others died in from $8\frac{1}{2}$ to $13\frac{1}{2}$ days. This series was likewise marked by the absence of any consistent difference in the time of survival between controls and experimental animals.

A small series of experiments (six animals) in which the course of infection with a highly virulent diphtheria strain² was studied in normal mice and in mice which had been either injected with India ink or splenectomized, disclosed no difference of susceptibility, between the normal and experimental animals. It was found that

² This strain killed guinea pigs weighing from 230 to 280 gm. in a dose of 1/20 slant within from 2 to 3 days.

doses as large as a whole slant of a 24 hour culture on Loeffler's medium were ineffective in producing in either group of animals any symptoms more marked than a small nodule at the site of injection.

DISCUSSION.

The results of the experiments described indicate that such elimination of the reticulo-endothelial system in the mouse as is accomplished by means of one blocking injection of India ink, or splenectomy, or a combination of both operations, has no influence on the natural resistance which this species possesses against diphtheria intoxication and infection. The conclusion which suggests itself is that in this case the mechanism of natural immunity differs fundamentally from the processes of artificially acquired immunity.

SUMMARY AND CONCLUSIONS.

1. The minimum amount of diphtheria toxin which killed normal mice of from 24 to 30 gm. in weight upon intravenous injection, was found to be between 75 and 100 times the M.F.D. for the guinea pig. When given intraperitoneally, the fatal dose for mice was as high as 200 M.F.D.

2. There was no significant difference in the lethal action of diphtheria toxin for normal mice and mice in which an elimination of the reticulo-endothelial system had been attempted by means of blocking injections of India ink, or splenectomy, or a combination of both operations.

3. Attempts to infect normal mice and mice treated as described with large doses of a highly virulent diphtheria strain were unsuccessful with both groups of animals.

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A STUDY OF THE PERIVASCULAR TISSUES OF THE CENTRAL NERVOUS SYSTEM, WITH THE SUPRAVITAL TECHNIQUE.

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PLATE 24.

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It has long been the accepted view that the ectodermal elements of the nervous system are separated by a compact membrane from the mesodermal tissues which accompany the blood vessels into the mass of the brain. Nissl (p. 345) held that this membrane, the *membrana limitans glia*, can be penetrated by mesenchymal cells only after it is damaged by the action of a severe pathological process.

Recently, however, del Rio-Hortega has questioned this view. Using the so called "silver carbonate" methods, which he has developed, he has found that a special manipulation of the technique brings out certain small cells which he terms the "microglia." In the adult, he finds these cells mingled everywhere with the ectodermal tissues of the brain. In the new-born animal, they seem to be concentrated in certain vascular zones of invasion, and from these regions he traces their wanderings into the ectodermal parenchyma. He brings evidence to show that these cells are the phagocytes of the nervous system, that they are identical with the phagocytes of the whole body, and that despite the fact that they seem to mingle freely with the ectodermal elements of the adult brain, they are none the less mesodermal in origin. Many workers, however, are still unconvinced as to the mesodermal nature of these cells. For this reason it has seemed desirable to apply to the central nervous system the supravital technique with which the phagocytic cells of the blood and of the general connective tissues of the body have recently been studied so minutely.

Vital methods have been used in the study of the phagocytes of the

central nervous system ever since the work of Bouffard, and of Goldmann. MacCurdy and Evans, and Macklin and Macklin used trypan blue, injected intravenously or intraperitoneally, to reveal the presence of dye-filled phagocytes in the brains of monkeys suffering from poliomyelitis and of rats subjected to sterile puncture wounds. The studies demonstrated that the phagocytes around brain lesions can take up particulate matter which reaches them through the vascular system. The nature and origin of the cells themselves are not settled by this observation and can be more directly determined by supravital methods.

EXPERIMENTAL.

The supravital technique has been applied to a series of sixteen normal animals, of which five were new-born rabbits, five guinea pigs under 7 days old, and six adult guinea pigs. Sterile puncture wounds were produced with a hot needle in four young anesthetized adult rabbits and in two guinea pigs; and an herpetic encephalitis was set up with herpetic virus of the strain "H.F." (described by Flexner and Amoss), by corneal inoculation in seventeen guinea pigs and twelve rabbits. After the supravital examinations were completed, all brains were fixed for subsequent study of stained sections. In this way was sought the ruling out of spontaneous disease in the "normal" animals while the extent of the lesions in the pathological material was established.

The supravital technique consists in the examination of the reaction of living cells to vital dyes immediately after removal of the cells from the body. Vital dyes, *i.e.*, neutral red and Janus green, were supplied to the cells in two ways: (a) In most of the experiments, fine films of dry neutral red and Janus green were prepared on slides according to the method described by Sabin, and by Sabin and Doan. When a minute amount of living tissue is placed on such a slide, the dye goes into solution in the tissue juices and reacts with the cells. (b) In six experiments, 300 to 500 cc. of dye (diluted 1:10,000 in physiological saline) were injected slowly through the aorta, after opening the cranium and the dura under anesthesia. In this way the brain could expand and become saturated with the dye, making it as certain as possible that the dye would reach every cell which had the power to react with it.

In obtaining the fragments of brain tissue, a standard technique was employed: (1) The brain was exposed under ether anesthesia. (2) The great vessels of the neck were opened to allow the brain to exsanguinate as completely as possible, so that the bits of brain tissue which were to be examined would be practically free from cells of the blood streams. (3) The dura and the pia-arachnoid were stripped away.¹ And (4) bits of brain tissue, about the size of a pin-head, were lifted out either with fine curved scissors, a sharp knife, or a capillary pipette. The bit of tissue was lifted out with as little damage as possible, without grasping with forceps, and was transferred gently to the slide which carried the film of dye. Under oil immersion magnification, the study of the preparation was undertaken at once in a warm box at body temperature. (5) In the meantime, the rest of the brain and some of the spinal cord were placed in a moist chamber in the warm box; and from time to time additional preparations were made, until an examination of as many regions as possible had been made. It was found that the brain would show little change under these conditions for nearly 2 hours, but that after this the new preparations would exhibit granulation, diffuse cellular and nuclear staining, and globules of free myelin. Before this point was reached, therefore, the brain was placed in a fixative.

DESCRIPTION OF OBSERVATIONS.

If a supravital preparation is made of a body fluid, it will spread itself under the cover-slip by capillarity. In this way blood (Sabin; Sabin, Cunningham, Doan, and Kindwall) lymph (Kindwall), cerebrospinal fluid (Kubie and Shults), peritoneal fluid (Sabin, Doan, and Cunningham, 1924), lymph gland, spleen, and bone marrow punctures (Cunningham, Sabin, and Doan, 1924, 1925; Doan; Sabin, Doan, and Cunningham, 1925) have been studied. Material taken from the brain, however, is too solid to spread without help, so that very gentle pressure must be applied, flattening the tissue out slowly and with a minimum of disruption. A preparation of this kind is always uneven. Some areas remain too dense for analysis; while in others the cells become entirely separated from one another. Of these separated cells, many remain morphologically undamaged, and are then particularly easy to study; but if injury occurs, it is readily recognized by the marked granulation of the cytoplasm and the rapid diffuse staining of the nucleus. (This usually happens around the edges of every preparation.) Many areas remain, however, which are neither too thick

¹ This is essential since, in a previous study (Kubie and Shults), these membranes have been found to contain phagocytes normally.

nor too thin, but in which the relations of various structures are well preserved, while the material is nevertheless thin enough for accurate observation under the highest oil immersion magnifications. And it is in portions of the preparations which have maintained their integrity in this way that the present observations have been made.

In these favorable areas, one special advantage of the supravital spreads is that the vessels and their surrounding tissues can be studied unshrunk and in their three-dimensional entirety, instead of in the single plane of a thin section of fixed material.

In the brains of normal animals, the perivascular meshwork contains at least two strains of cells which are readily recognized in the supravital preparations, the lymphocyte and the clasmatocyte. Lymphocytes are recognizable in three sizes: small, intermediate, and large. They take up little neutral red, and that in small vacuoles around the periphery. They have characteristic mitochondria, which vary from long, rod-shaped structures in the larger cells, which are probably the younger forms, to round dots in the smaller cells. They have a type of motility which is peculiar to themselves, in that they push their nuclei ahead of them, with a zone of thinner cytoplasm around the nucleus and the rest of the cytoplasm tailing out behind. This motility is observed chiefly in the intermediate size group.²

In contrast to the lymphocyte the mitochondria of the phagocyte are relatively obscure; while the vacuoles are abundant and prominent, and fill with neutral red in the presence of the dye. The dye in the vacuoles is colored from pale lemon-yellow to deep orange-red, indicating a pH range of approximately 6.8 to 8.0. The significance of these changes in color are discussed in a forthcoming paper by Sabin and Doan. It is only in an occasional cell that the differentiation of the lymphocyte from the phagocyte presents any difficulty, such that one might hesitate between calling a cell a heavily stimulated lymphocyte or a small and inactive phagocyte. In general, the larger size of the phagocytes and their dye-filled vacuoles make them one of the most unmistakable cells in the body, as will be readily seen in the illustrations which follow.

² Evidence has been presented elsewhere (Kubie and Shults) that this motile lymphocyte is the plasma cell of Unna.

Two morphological groups of phagocytes have been described (Cunningham, Sabin, Sugiyama, and Kindwall; Sabin and Doan; Sabin, Doan, and Cunningham, 1924, 1925) and the subject of their functional and cytogenetic differences has become the object of vigorous inquiry and argument (Lewis and Lewis). One of the groups has been called, after Ranvier's original term, the "clasmatocyte" (Sabin, Doan, and Cunningham, 1924) and the other is the monocyte. Of these two phagocytic forms, only the clasmatocytes are illustrated and discussed here, as they are the characteristic mesodermal phagocytes of the perivascular sheath under these experimental conditions. Monocytes have been seen in only two of the present series of experiments; and since in a previous study (Kubie and Shults), young monocytes were seen to form a normal component of the arachnoid, it is possible that in these two experiments the monocytes may have arisen from bits of arachnoid which were unwittingly included in the preparations.

In Fig. 1, a typical resting clasmatocyte is seen against the endothelial cell of a venule from the occipital cortex of a normal young guinea pig. The cell is stretched along the venule, closely applied to its wall. In other places, as in Fig. 4, it may be seen to stretch along the outer border of the perivascular channel, but in the preparations from normal animals it is always elongated and never rounded up. Even in the normal, however, these cells may be quite abundant. This is shown in Fig. 2, which shows the maximum number of such cells which have been observed in normal material, in this instance taken from the parietal cortex of another young guinea pig.

The contrast between the clasmatocytes of the normal brain and those which show the effect of infection is well illustrated in Fig. 3. Here, the cells appear swollen, often rounded up, with increased vacuoles, and probably an increase in actual numbers. The picture is drawn from an arteriole from the parietal cortex of a young rabbit, at the height of an encephalitis which had been induced by corneal inoculation with herpes virus 6 days before. It will be noted that in this picture two clasmatocytes are seen lying free from the vessel wall. While this is not an unusual observation, it is not the rule; and it is not safe to conclude that the clasmatocytes are able to invade the surrounding brain tissue when in the body, because it is possible that the process of spreading the material could dislocate a few cells, even where the majority have preserved their architectural relationships intact. The clasmatocytes which are occasionally seen free from any vessel wall appear to lie *on* rather than in the ectodermal parenchyma.

In Fig. 5 there is represented the phagocytosis of trypan blue in clasmatocytes along a small vein from deep within the occipital cortex of a young rabbit. This rabbit, over a period of 7 days, had been given three small subarachnoid injections of 1 per cent trypan blue through the cisterna.

Turning to the lymphoblastic group of cells, it was found that small nests of these cells occurred regularly in the normal animals. One such nest is illustrated in Fig. 4, from the cornu ammonis of another normal guinea pig, in the fork formed by the juncture of two venules. Such nests of lymphoblastic cells, containing large, intermediate, and, more rarely, small forms, have been found repeatedly in forks such as the one illustrated. In sections, only a small fragment of such multi-layered relationships as these can be demonstrated. Along the unbranched lengths of straight vessels, single lymphocytes, or short chains of three or four in a row, are regularly seen, scattered along the sheath.

The lymphocytic response to disease has been difficult to demonstrate intact, because the larger proliferations are easily ruptured and spill their contents diffusely over the preparation. In Fig. 6, however, is seen the striking multiplication of these lymphoblastic forms in all stages of development. This point of intersection of two venules is from the parietal cortex of an encephalitic rabbit, and at this point it is possible to note a characteristic phenomenon, namely that where the lymphocytic response is intense very little clasmatocytic reaction seems to occur. This is illustrated again in Fig. 5. There is drawn here only one-half of what was actually a long, cigar-shaped dilatation of the vessel sheath. At one end of this fusiform swelling (the end not included in the drawing) were clasmatocytes engorged with trypan blue and quite identical with those pictured at the other pole. In between the two points of intense clasmatocytic response were masses of proliferating lymphocytes which made a cellular tube around the blood vessel, a structure too complex for adequate reproduction. Where the clasmatocytes lie, the sheath is narrow and clings closely around the vessel. Where the lymphocytes proliferate, the sheath is distended, and in it are found only the filmy ends of the clasmatocytes whose main bodies lie at the two poles of the swelling. The stimulation or response of the clasmatocytes and

lymphocytes never seems to occur quite at the same place, but always with a marked preponderance of either one form or the other at any one point.

DISCUSSION.

1. In these studies it has been possible to demonstrate that in the adventitial sheath of the blood vessels of the central nervous system of normal guinea pigs and normal rabbits, both lymphocytes and mesodermal phagocytes are always present, a finding which is opposed to the view of Nissl (pp. 319 and 340), who denied the presence of lymphocytes in normal brains.

The question must be considered as to whether the occurrence of lymphocytes and phagocytes does not from the outset throw doubt on the "normality" of the brains in which they are found, despite the absence of any outspoken evidence of disease. With laboratory-bred rabbits or purchased stock kept under observation, it is always uncertain whether the so-called spontaneous encephalitis exists or not. The condition is usually asymptomatic, and the proof of its occurrence is supplied by postmortem histological examination. Many of the animals used in the present experiments, however, were laboratory-bred and very young. In such animals, these cells have been found just as plentifully in the rabbits and guinea pigs which were under a week old as in the later stages. Furthermore, for the observations on animals more surely normal, most dependence has been placed upon the guinea pig, which almost never suffers from spontaneous encephalitis. And in every animal reported as normal, study of sections from all parts of the central nervous system has revealed no foci of active perivascular infiltration, no neuroglia scar formation, and no changes in the neurons. The absence of histopathological evidence of disease, and the presence of the cells from birth seem to justify the conclusion that the animals were normal.

2. It is at present possible only to suggest the relation which the clasmotocytes of the perivascular sheaths may bear to the microglia of del Rio-Hortega, or to the compound granular corpuscles or *Gitterzellen* of classical neuropathology. If the view is true that an impenetrable barrier separates everywhere the mesodermal from the ectodermal tissues of the brain, then the clasmotocytes cannot be the

SUMMARY.

1. The presence of clasmatoocytes in the adventitial sheath of the blood vessels of the normal brain has been shown. Thus far, these are the only mesodermal phagocytes, the presence of which in the central nervous system has proven to be demonstrable by the supravital technique.

2. The clasmatoocytes are vacuolated even normally.

3. The vacuolation increases in the clasmatoocytes of the whole brain as result of a localized injury, and it increases also throughout the central nervous system in the early stages of an herpetic encephalitis, even before gross damage to nerve cells or myelin can be demonstrated.

4. The endothelial cells of blood vessels have not exhibited any signs of multiplication or of vacuolation. They have not apparently given rise to any of these phagocytes, nor taken any part in the phagocytic reaction themselves.

5. Along capillaries, the phagocytic cells are seen only rarely, and it is possible that they have wandered along these vessels from the nearby venules and arterioles.

6. Trypan blue, injected without pressure into the subarachnoid space, finds its way rapidly down the perivascular sheath and enters the clasmatoocytes of the sheath, deep within the substance of the brain. From this fact may be deduced the functional patency of the perivascular channels and their continuity with the subarachnoid space.

7. Lymphocytes are seen normally in the perivascular sheath, and the presence of the large young forms along with the intermediate and the small old cells suggests that they develop in this situation and give rise to the lymphocytes of the cerebrospinal fluid by wandering out into the subarachnoid space.

8. In response to an herpetic encephalitis, as well as other injury, a huge increase in the number of lymphocytes and of clasmatoocytes occurs in the perivascular tissues.

9. The multiplication of both cell types seems to begin simultaneously, but never at the same spot, any one point along a vessel exhibiting a marked preponderance of either one form or the other.

10. The resting clasmatoocytes cannot readily be differentiated from

any other fusiform cell in sections of fixed tissues; while the lymphocyte may appear, in such sections, as an apparently isolated small round mononuclear cell, with no visible relation to the perivascular apparatus.

CONCLUSION.

By means of the supravital technique, it has been found that the perivascular tissues of the brain contain normally two types of cells—lymphocytes and clasmotocytes. It is believed that these cells are always present and proliferate *in situ*, both under normal and pathological conditions.

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EXPLANATION OF PLATE 24.

FIG. 1. Clasmatocyte against the endothelial cell of a small vein, supravitally stained with neutral red. From the occipital cortex of a normal guinea pig (K 51). \times about 500.

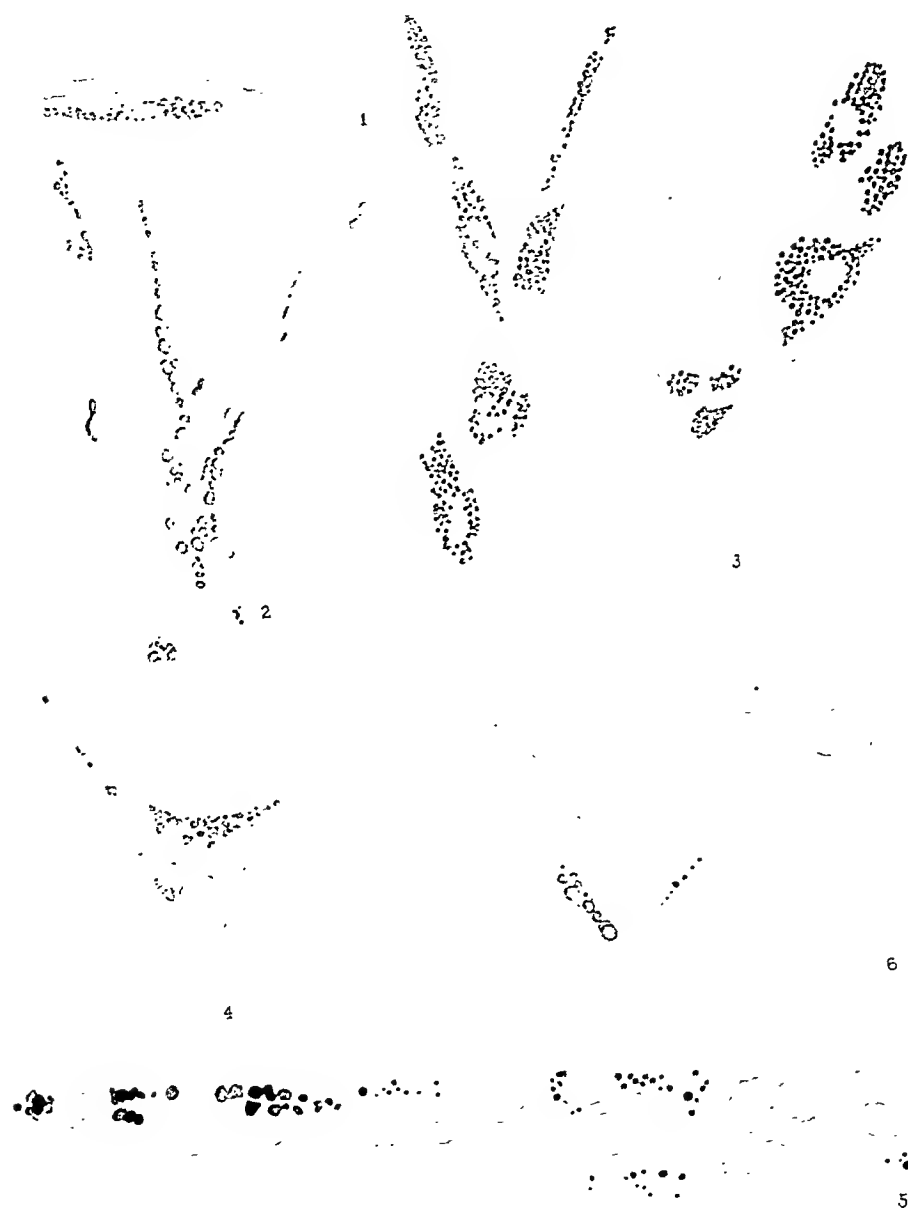
FIG. 2. Clasmatocytes stretched along the walls of a branching group of capillaries and venules, supravitally stained in neutral red. From the parietal cortex of a normal guinea pig (K 50). \times about 600.

FIG. 3. Clasmatocytes along the walls of arterioles, rounded up in response to the stimulus of an herpetic encephalitis. Supravital staining with neutral red. From parietal cortex of a rabbit (K 53). \times about 500.

FIG. 4. Lymphoblastic nest in the perivascular sheath at the junction of two venules, from the cornu ammonis of a normal guinea pig (K 51). Lymphocytes unstained. Clasmatocyte with vacuoles filled with neutral red lies stretched along the outer border of the perivascular sheath. \times about 500.

FIG. 5. Reaction of lymphocytes and clasmatocytes to subarachnoid injection of trypan blue. From the occipital cortex of an otherwise normal rabbit (K 74). Trypan blue appears black in the reproduction. \times about 500.

FIG. 6. Proliferating lymphocytes at the intersection of two venules from the corpus striatum of a rabbit with herpetic encephalitis (K 68). \times about 500.



THE RELATION OF MONOCYTES AND CLASMATOCYTES TO EARLY INFECTION IN RABBITS WITH BOVINE TUBERCLE BACILLI.

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PLATE 25.

(Received for publication, June 20, 1927.)

In a recent paper, Lewis and Sanderson (1) reported the differences in the reaction in the lungs of rabbits to intravenous injections of human and bovine tubercle bacilli in doses of 1 mg. They studied the material in fixed sections, killing the animals at intervals of 48 hours beginning 2 days after the inoculation. It was found that the response was the same to the two strains of bacteria for the first 8 days, and that then there was a rapid disappearance by absorption of the tubercles in the animals infected with the human strain, in contrast to their continued progressive increase with the bovine organisms. On the 2nd day after infection, they found in all animals a marked thickening of the interalveolar septa of the lungs, due in part to congestion and in part to an increase in mononuclear cells of the "endothelial type," and to polymorphonuclear leucocytes, both amphophilic and eosinophilic. They found small tubercles present from the 2nd to the 4th days.

We have repeated these experiments with rabbits, using the bovine strain of tubercle bacilli only, and making studies of the tissues at intervals of 24 hours for the first 4 days and thereafter every 48 hours throughout the acute reaction of the 1st month. The tissues of the lungs, the liver, the spleen, the mesenteric lymph glands, the omentum, and the bone marrow were studied routinely in the fresh, with the supravital technic, before fixation.

The Lungs.

24 hours after the injection of the bacilli (R 115) no increase in the free cells that come from a gentle scraping of the fresh cut surface of the lung could be

detected. As in the normal, the free cells present were largely of two types, clasmatoocytes and monocytes, though there were already present occasional true epithelioid cells, and areas of primitive cells (2) with typical mitochondrial distribution and no reaction to neutral red. Examination of representative areas near the hilum of the lung showed both clasmatoocytes and monocytes with the usual phagocytosis of carbon, the former containing it in large masses, the latter, together with an occasional epithelioid cell, storing it in fine scattered granules. While these types of cells were found in relatively the same proportions in other parts of the lungs, notably the apices, the content of carbon was quantitatively less. The polymorphonuclear leucocytes were present in numbers only representative of the circulating blood in the organ.

At 2 days (R 116 and R 215), just as Lewis and Sanderson found, there was an increase in free cells due in large part to clasmatoocytes. The preparations all showed an excess of debris and, perhaps correlated with this, an increase of leucocytes. Some primitive cells and monoblasts, many very young monocytes, an occasional epithelioid cell, and lymphocytes were present. At 72 hours (R 120) there were many more small lymphocytes than previously seen, but with clasmatoocytes still predominating in the large mononuclear group. From the apex was revealed an area of typical epithelioid cells covering about four oil immersion fields in the microscope, the first appearance of associated epithelioid cells, the earlier reaction being manifested in scattered individual cells only. This beginning formation of tubercles was confirmed in the stained sections of the fixed tissue.

On the 4th day (R 216) the reaction had become predominantly monocytic; many young monocytes were found together with epithelioid cells and typical giant cells of the Langhans type, and certain of the epithelioid cells showed the beginning development of refractile, fat bodies in the periphery. In certain areas the clasmatoocytes loaded with massed debris still were very numerous. In supravital surveys, at least, it seems essential, in describing the reaction of the tissues to tubercle bacilli, to differentiate two types of large mononuclear cells. While present together in all preparations, epithelioid cells and clasmatoocytes tend to react ultimately so differently to neutral red, their concentration of vacuoles and debris is so dissimilar, and their varying percentages in the course of the development of the special pathology of the disease is so striking, that it is found convenient and necessary to differentiate them in the attempted description of the pathological lesions and their progress. This will be discussed in detail in the latter portion of the paper.

From the 6th to the 18th days (R 217, R 218, R 227, R 228, R 229, R 230, and R 231) the lungs showed an ever increasing limitation of air-containing alveoli coincident with a progressive consolidation with epithelioid and giant cells. Always there were present, however, many young monocytes; at least in one interpretation, the *sine qua non*, the source and origin of the increasing epithelioid cells. Together with the constant presence of the clasmatocytic scavengers

throughout this period, there was evidence of abnormal desquamation of bronchial and alveolar epithelium in the supravital spreads.

The Liver.

No reaction was noted in the liver until the 6th day after intravenous infection, when a few scattered epithelioid cells and one typical giant cell were found in the supravital surveys. The Kupffer cells appeared normal in number and activity. On the 10th day was noted a decided increase in number and activity of the Kupffer cells, some fields showing 18 to 20 cells of this type. There were present many monocytes and occasional epithelioid cells. In one area there was a circumscribed group of 10 to 12 stimulated monocytes looking like a young tubercle. Only limited localizations of epithelioid cells have been observed in the liver of the rabbit under the conditions of this experiment during the 1st month after infection.

The Spleen.

The spleen revealed nothing abnormal in the supravital surveys for the first 72 hours. On the 4th day, however, this organ was definitely enlarged (weight 1.5 gm.), the lymphoid follicles standing out sharply on the slightly bulging cut surface. The clasmotocytes were increased in number; no epithelioid cells were found. On the 6th day the weight was 1.7 gm. On the 8th day there were found primitive cells which were interpreted as monoblasts, and the sections of fixed material on the 6th and 8th days showed a few initial small foci of epithelioid cells. At 10 days, the weight of the spleen was 3.1 gm.; color, deep red; acute splenic tumor, with sinuses engorged with blood. Supravital studies at this time showed increasing monocytes with typical epithelioid cells; clasmotocytes in large numbers contained their usual complement of red cells plus much finer debris. On the 12th day were found scattered everywhere small clumps of epithelioid cells (spleen weight 3.2 gm.) in addition to the clasmotocytes heavily engorged with all kinds of red cell debris. Sinuses were dilated with blood.

The spleen at 14 days weighed 4.9 gm., being dark maroon in color. The clasmotocytes showed marked phagocytosis of both red and white cells, indicating an injury predisposing the latter to ingestion. A few scattered clumps of epithelioid cells were present; sinuses congested.

On the 16th day the spleen weighed 3.5 gm., was a deep red, and presented a moist cut surface. There were from three to five epithelioid cells per oil immersion field and numerous typical giant cells; the lymphoid tissue appeared to be relatively less in amount, some myelocytes were seen, and an occasional large megacaryocyte with massive cytoplasm was observed; the usual clasmotocytes, highly phagocytic, were prominent. On the 18th day the spleen weighed 4.2 gm.

From the 4th day after infection all rabbits surveyed during the 1st month have shown the development of an acute splenic tumor, largely due to dilated

sinuses, increased numbers of actively phagocytic clasmatoocytes, and from the 8th to the 10th days increased monoblasts, monocytes, and epithelioid cells, with tubercular foci in limited number scattered both in follicles and parenchyma.

The Lymph Glands.

In the study of the mesenteric lymph glands during the first 18 days after infection, the results have been constant. Beginning with the 4th day these glands have all shown a marked edematous distention due to a great increase of fluid in the lymphatic sinuses. On section of the fresh gland there was a copious flow of opaque fluid having a tendency to coagulate quickly. The supravital preparations were made by taking a drop of this fluid, with and without scraping the cut surface of the gland, the mount being made as with a film of blood. The early reaction as revealed by these preparations was wholly clasmatoeytic though only a few intact phagocytes were found. In addition to the usual lymphocytes, mostly small, there were great masses of extracellular stained debris, identical in appearance with that found in the intact clasmatoocytes. In conformity with the known fragility of these cells when highly phagocytic, the interpretation that there was great activity of the clasmatoocytes or endothelium of the sinuses, and that many of these cells had disintegrated, seems justified (3).

On the 6th day there was found for the first time in addition to the above an extensive stimulation and development of very large young cells, difficult of differentiation between monoblast and lymphoblast at this stage in this environment; many oil immersion fields of the microscope showed as many as 20 such cells. The rabbits representative of the period from the 6th to the 12th days presented much the same picture as just described. On the 12th day, in addition to many young monocytes, typical true epithelioid cells were found, both singly and in small foci, which finding was typical of the glands examined from this date on in the acute period of the disease.

These observations agree in general with those of Kageyama (4) who made studies of the lymph glands of mice after intraperitoneal inoculation. He found the tubercle bacilli free in the sinuses after 5 minutes, and within the lining endothelial cells in 15 minutes. These endothelial cells rounded up and became free and within 2 hours could be shown to be filled with bacilli. The cells died in from 6 to 18 hours, the leucocytes then coming into activity. The leucocytes in turn died and were phagocytized by new histiocytes; after the disintegration again of the latter no bacilli could be stained. Only after the 7th day were the epithelioid cells of tubercles found, containing very few bacilli.

These combined observations indicate the marked phagocytic activity of the clasmatoocytes for tubercle bacilli, the fragility of the cells, but their apparent power in at least destroying the acid-fast characteristic of the organism. Also, in contrast to the lung, the late development of tubercles in lymph glands is indirect evidence against the normal presence of monocytic foci in the latter.

The Omentum.

The omentum lends itself admirably to supravital surveys, because the relationships of all cells are maintained in the thin films of this tissue, which it is possible to make on slides prepared in the usual manner. Until the 4th day the omentum showed only the usual clasmotocytic and fibroblastic distribution of cells. On the 4th day there appeared in addition a few, scattered, young, developing monocytes, and the clasmotocytes from this stage on showed increased phagocytic activity and were predominant in their numerical strength up to 12 days. On the 8th day many clasmotocytes in the omentum contained acid-fast debris. On the 14th day epithelioids had appeared among the increasing monocytes and definite aggregations of epithelioid cells in tubercles were found on the 16th day.

The Bone Marrow.

The reaction of the bone marrow is initiated on the 8th to 10th days by the development of large numbers of young monocytes *in situ*, followed from the 12th to the 20th days by an increasing encroachment on fat cells and hemopoietic foci by typical tubercular tissue. This process is followed in detail in an earlier paper in this *Journal* (5).

Thus it will be seen that with the intravenous portal of entry the initiation of the tubercular process in the lungs definitely antedates that observed in the other organs of the body as analyzed in these supravital surveys and confirmed by study of fixed sections. The first epithelioid cells are found in the lung within 24 hours after the injection of tubercle bacilli but not until the 6th to the 14th days in the other tissues studied. The earliest and most obvious reaction in all organs is the clasmotocytic response, and the development of the tubercular tissue, epithelioid and giant cells, is always preceded or accompanied by a stimulation of monoblasts and young monocytes, apparently *in situ*.

There is at present much difference of opinion concerning the phagocytic mononuclear cells, the prevailing idea being that they constitute a single strain. The recent studies by the method of tissue culture by Carrel and Ebeling (6), and by Lewis and Lewis (7) have brought strong support to this view.

Carrel and Ebeling studying monocytes from the blood and macrophages from the connective tissues found that the monocytes taken directly from the blood of the animal were smaller than the macrophages but that this distinction disappeared with proper nourishment in tissue culture; that monocytes and macrophages grew well with the same nutrient elements in contrast to fibroblasts; that they were

equally affected by certain toxic agents; and that they had the same type of actively waving surface films. From these common properties they have concluded that any morphological differences between the two types are due to environmental influences. Lewis and Lewis have made extensive comparative studies of blood beginning with the fish and have also concluded that monocytes and clasmotocytes are a common strain. Neither of these studies with tissue culture has taken into consideration the fact that the desquamated endothelial cell, often a degenerating type it is true, but also quite as often a living, active cell, is present together with the monocyte in normal blood (8). Moreover, even as blood cannot be considered the source of a pure strain of monocytes, neither can the connective tissues be considered the source of a pure strain of clasmotocytes since both clasmotocytes and monocytes are present in connective tissues. However, these experiments with tissue culture are a great advance in our knowledge of the phagocytic mononuclear cells. Though there is considerable evidence to show that these two strains of cells do vary independently in their response to certain pathological conditions, nevertheless Carrel and Ebeling have definitely proved that under the conditions of tissue culture they both respond alike to certain stimuli.

The history of the type of cell now discriminated as the monocyte, distinct on the one hand from the granulocyte and on the other from the lymphocyte, is a confused one. The monocyte was first considered in the Ehrlich classification as a transition form in the maturation of the leucocyte. This error involved the idea that the specific granulation of the monocyte was of the neutrophilic type. The correction came with the introduction of the Romanowski methylene blue-azur, specifically with the use of azur. With this technic, it was discovered by Michaelis and Wolff (9) that both lymphocytes and monocytes (transitional leucocytes) contained azur granules. That the granulation of the monocyte has no relation to the neutrophilic granulation was fully demonstrated by Pappenheim and Ferrata in 1911 (10). The monocyte was described as making a third strain of the white cells of the blood, distinct from leucocytes and lymphocytes, by Schilling-Torgau in 1912 and 1914 (11), and since then this interpretation has been generally recognized and accepted by hematologists.

However, the literature, particularly the clinical, has continued, even to the present time, to give records of blood counts in which monocytes and lymphocytes are recorded together as mononuclear cells without differentiation. The confusion attendant upon the lack of recognition of these cell differences clinically may well be illustrated in the disease known as infectious mononucleosis. Unpublished observations by both of the present writers in independent studies at the Johns Hopkins Hospital and the Boston City Hospital confirm the lymphocytic, rather than the monocytic, nature of the mononuclear increases in this condition.

The discrimination of lymphocytes and monocytes can be made in a good methylene blue-azur stain. The cytoplasm of the monocyte reacts with a dense muddy blue color in which are embedded in the human strain varying numbers of tiny azurophilic granules (12), not identical with the azur granules of lymphocytes, since they are smaller and stain darker. The clearness with which these tiny granules may be seen is a good gauge of the success of the stain and the adequacy of the lens used. If the stain be too basophilic, the granules will be obscured.

In the fourth edition of his text-book, Naegeli (13) states that these granules of the monocyte can be seen with dark-field illumination in the living cell. This we have confirmed. Since the monocyte has great numbers of tiny mitochondria, however, it is necessary to identify them also with the dark-field illumination and to discriminate them from the specific granulation. If a film of blood be stained with neutral red and Janus green and studied with a microscope in which first direct and then dark-field illumination can be applied to the same cell, it is easy to demonstrate with a lymphocyte that the mitochondria, stained green, but refracting pink in dark-field illumination, are the only bodies visible. By selecting a monocyte in which there are but few neutral red vacuoles and in which the mitochondria are well stained, the cytoplasm of the human monocyte under the dark-field appears fully as granular as the neutrophilic and the eosinophilic leucocytes themselves. This granulation, seen with the dark-field, in the human monocyte is neither the same substance as the bodies that react to neutral red in the living cell nor the mitochondria, and is, therefore, by one more criterion differentiated

from the myeloid granulation. In the monocyte of the rabbit, there is a marked reduction of the azurophilic granulation in fixed films, most of the cells showing none at all, and the granulation visible in dark-field illumination suffers a corresponding reduction. In a more recent review, Naegeli (12) gives a plate of human monocytes showing the variations in the content of azurophil granules down to a cell, representative of the occasional human monocyte, which lacks them entirely. The latter is entirely comparable with the usual monocyte of rabbit blood, the basic muddy blue cytoplasm typical of all cells of this type being independent of species and granulation. With reference to this strain of cells in the two species, the peroxidase reaction has been found (14) to parallel the azurophilic granulation qualitatively and quantitatively, the majority of human monocytes giving a positive oxidase reaction, the opposite being true of the rabbit monocyte.

An adequate and assured discrimination between lymphocytes and monocytes became much easier with the introduction of the supravital technic for the study of blood (15). When attention was focused on living blood cells studied in their reactions to neutral red and Janus green, distinctions between these two strains of cells, other than those above mentioned, became clear. The lymphocyte is characterized by a clear cytoplasm in which are many mitochondria and a few scattered vacuoles, which stain in neutral red, and which vary greatly in number and size according to the activity of the cell; these vacuoles in fresh preparations of normal blood are often entirely absent in the small forms. The nucleus is seldom round, but changes constantly in shape, especially as the cell moves. The type of motility is characteristic, and like that seen in no other cell; the nucleus is always in the front end of the moving cell and changes so rapidly in shape that it seems to take an active part in the locomotion of the cell.

The monocyte, on the other hand, both of human and of rabbit blood, shows a rosette of neutral red bodies of a uniform salmon tint, and great numbers of tiny mitochondria. The color and nature of the vacuoles reacting to neutral red are identical in all species, their relative number only influencing the sharpness of definition of the clearly maintained centrosphere. In the specific reaction to tubercular infection, the epithelioid and giant cells of rabbit and human

tissues are indistinguishable in their characteristic reaction to vital dyes, while obeying the species response to the peroxidase reaction (14); rabbit epithelioid, oxidase-negative, human epithelioid, oxidase-positive. The cells in locomotion are stretched out often in triangular shape; there is a slow progression with less active streaming of particles than with the granulocytes; in the supravital technic, the surface films do not have as free play, due to the narrowness of the space between slide and cover-slip, as in the tissue culture preparations. These distinctions of motility are fully demonstrated in the moving pictures of Carrel, Ebeling, and Rosenberger.

Thus, in the history of the study of the blood the monocyte was first confused with the neutrophilic leucocyte, this error being gradually corrected when it became clear that the monocyte never developed a neutrophilic granulation. The monocyte then came to be regarded as indistinguishable from the lymphocyte. But the azur granules of lymphocytes are large, few in number, and inconstant, while the azurophilic granules of the human monocyte are tiny and usually extremely numerous; the degree of basophilia of the cytoplasm is distinguishing; and the living cells show a different type of motility and react characteristically to the supravital dyes. Then, too, the differential response in pathological states has further separated the types (16).

We are now in a third stage in the history of the monocyte in its struggle for recognition as an entity, when it is by some regarded as identical with the clasmatoocyte of the connective tissues. It is clear that in the present day study of the mononuclear cells, the first step, as in the past, must be the effort to see if there are finer morphological discriminations by which different types may be distinguished, and, then, to see if such criteria may be of aid in finding out and analyzing any differential response of these cells in physiological and pathological conditions.

The morphology of a cell with such a wide range of phagocytic activity as the clasmatoocyte must be varied. The cytoplasm of the clasmatoocyte stained in methylene blue appears as a faintly basophilic thin film (8) in contrast to the dense cytoplasm of the monocytic strain. This contrast is shown in Figs. 8 and 9, which are clasmatoocytes, in contrast to Figs. 6 and 7, which are an epithelioid cell and

a giant cell of the Langhans type. The cytoplasm of the clasmatocyte has no specific granulation except for substances phagocytized. Hence, when granules are present, they may be of any size from tiny points to whole included cells, and they may react with any of the shades of the dyes used, according to the original nature of the substances taken in or the state of digestion in which they are found. Likewise, in the supravital technic, the clasmatocytes vary all the way from a small cell with a few vacuoles stained with the neutral red up to enormous cells engorged with whole cells and debris.

In general, there are two groups of clasmatocytes: first, the constantly active phagocyte of certain organs, the derivatives of the so called specific endothelia, namely of spleen, lymph glands, bone marrow, and liver; and secondly, the normally less active phagocytes of the general connective tissues.

With the supravital technic we have found that the normal monocyte of the blood and the resting clasmatocyte of the tissues (Fig. 3) are readily discriminated by the nature of the stained vacuoles. This much might be granted under either concept of their relationship, whether they are to be considered as different strains or simply as different phases of the same strain of cells. The vacuoles of the monocyte of the blood show a uniform salmon-red reaction to vital neutral red and they form a relatively permanent rosette around the centrosphere of the cell.

In the unstimulated clasmatocyte of the tissues, the vacuoles are not in a rosette but are scattered throughout the cytoplasm, and they may stain red, or orange, or yellow; moreover when the cell is watched in the supravital preparation, there is usually a tendency for the color, if originally red, to change toward yellow. When the monocyte is watched in the supravital film, the red color of the vacuoles tends to be retained persistently even to a stage of degeneration which involves a destruction of the cell membrane. This phenomenon is not seen unless the dye is in a concentration that is differential.

A dilution of vital neutral red to be discriminatory must be carefully adjusted. Fortunately, in normal blood, there is an excellent standard for the optimum range of neutral red as an indicator; the eosinophilic granules should show the alkaline or yellow reaction of one extreme, pH 8; the basophilic granules should show the other

extreme with the deep scarlet of the acid end, pH 6.8; the vacuoles of the monocytes should give the salmon shade of an intermediate pH. In rabbit blood with the high percentage of basophils and sufficient eosinophils, the standardization is readily made. Most of the neutral red in use gives a yellow tone modified by a coppery tinge for the eosinophilic granules. A perfect dye has only the yellow tone. Occasionally two different eosinophils in a drop of blood vary slightly in their reaction to the dye. The basophils must have the brilliant scarlet reaction. If the concentration of the neutral red is too great, every substance that reacts at all in the cells will be a deep uniform red. Usually in such a preparation a lessened motility of the cells will also indicate a toxic reaction of the dye. In a dilution that brings out the entire range of color from red to pure yellow, the vacuoles of the monocyte and epithelioid cell are distinctly and uniformly within the acid range of the dye but never as sharply scarlet as the reaction of the basophilic granules. We have described the characteristic shade of the vacuoles of the monocyte as a salmon-red. In becoming the epithelioid cell the vacuoles may assume a uniform carmine tone, as is shown in Fig. 2. The clasmatoocyte on the other hand quite as characteristically shows every range of color of the dye in the same cell, often all shades being present. The clasmatoocyte of Fig. 5 shows the orange and yellow tones.

Because the shade of the dye in the vacuoles of digestion of the clasmatoocyte frequently changes from the varying tones of red through orange to pure yellow while the cell is watched, the question is raised as to whether this change may not represent a physiological process fundamental to this type of cell, indicating that the development of a relatively alkaline reaction may be essential to the digestive processes of the vacuoles. On the other hand, this change in color may be only the less significant step in a process of degeneration and cell death. If the latter be true, then monocytes do not tend to degenerate in this manner.

Often the clasmatoocytes show the yellow reaction of the vacuoles when first taken from the animal. This was conspicuously the case with the stimulated clasmatoocytes of the lung the first 3 days after the injection of tubercle bacilli. These contrasts are well seen in cells of Figs. 1 to 5. Figs. 1, 2, and 3 are a fibroblast, an epithelioid

cell, and a clasmatoocyte taken from the omentum of a rabbit, R 160, which had received twelve doses of a phospholipin, designated A-3, isolated from human tubercle bacilli by Dr. R. J. Anderson. The full account of the experiment is given in an accompanying paper in this *Journal* (3). The three cells, Figs. 1, 2, and 3, were in the same field; the fibroblast was in the middle and the other two were not more than the diameter of two red cells apart. The red cell of Fig. 4 gives the magnification. The fibroblast was unstimulated and showed no reaction to the neutral red. The epithelioid cell of Fig. 2 is typical; it is the type of cell characteristic of the lesion of tuberculosis itself, in this instance produced in response to a chemical factor from the bacillus. It shows the carmine reaction of the tiny vacuoles of the rosette and this color showed no tendency to fade on standing. The clasmatoocyte, Fig. 3, is the typical long cell characteristic of the resting type to be seen in the normal omentum and in the general connective tissues. When this cell was first seen, its vacuoles had considerable red but the epithelioid cell was drawn first and by that time all the vacuoles of the clasmatoocyte were yellow as shown in the drawing and yet there was no indication of any damage to the cell. The omentum provides perhaps the best material for supravital studies in the early stages of a peritoneal irritation, because it is so thin a film that one can, so to speak, dissect the entire thickness with the focusing screw of the microscope, and because the cells are retained in their original relative positions. The position of these three cells, all within one oil immersion field of the microscope and all three showing a different reaction to an identical concentration of the same dye, is evidence toward the view of a differentiation of the cells of the connective tissues in an environment that must be essentially the same for each. It is difficult to imagine any great difference in environment between these three cells as far as soluble factors are concerned. The phospholipins of the experiment were not in solution but in the form of an emulsion, but there is evidence that the clasmatoocytes also take in lipoids. If, then, both types of cells take in the lipoids, it appears that they treat this material differently.

The cell of Fig. 5 was in our interpretation a clasmatoocyte from the circulating blood of a rabbit, R 193. This animal was one of a group of six animals which were inoculated with 1 mg. of a culture

of Strain B1, which had been growing for 2 months and proved to be largely attenuated. The signs of tuberculosis were slow in developing; there was no anemia in a month, and in only two of the animals was there any increase in monocytes in the blood. The constant reaction of the blood during the 1st month to an active culture of this strain (B1) has been reported in another communication (5). A second dose of 2 mg. from a fresh culture of the same strain of bacilli was given 1 month after the first injection. The next morning five of the six animals were dead; two of them were still warm so the living cells could be studied, and the reaction in all the tissues with the supravital technic was of the clasmotocytic strain. In the sixth animal, that survived, there was a rise in clasmotocytes to 1600 per c.mm. on the next day, there were 1200 cells too far degenerated to be classified, while the monocytes showed only 800 per c.mm. of blood. The cell of Fig. 5 was drawn during this rise (see Chart 5 of a preceding paper (5)). It will be noted that the cell was heavily vacuolated with orange as the predominating shade, and during all the time that the cell was being drawn the color was changing toward the yellow tones. This slide was kept for 3 to 4 hours and the monocytes still retained their red vacuoles long after all color had faded from the clasmotocytes and they had disintegrated. We have discussed elsewhere (8) the fragility of the clasmotocytes in supravital preparations and their tendency to a sudden bleaching of all color at the time of the death of the cell. In this series of experiments, every lymph gland studied in the acute reaction to a massive injection of tubercle bacilli showed debris which had probably come from the disintegration of clasmotocytes. The debris found in fresh scrapings of the lungs 2 days after inoculation may have been from the same source since the only marked increase in cells at that time was in the clasmotocytic strain. An excess of cellular debris is also frequently found in the peripheral blood of the rabbits during tuberculosis.

In the reaction to neutral red, the monocyte shows an almost uniform color of the vacuoles in a given cell and the shade is of a salmon tint which may become a carmine tone in the tiny vacuoles of the epithelioid cell. The color of the vacuoles of the clasmotocyte varies all the way from red to yellow in one cell, and there is every possible combination of shades with marked changing toward yellow in many

cells. It is clear, then, that while there is an overlap in color reaction of these two strains toward the acid range of the dye, only the clasmatoocyte tends toward an ultimate alkaline reaction.

It has been brought out by Lewis (17) that the arrangement of vacuoles in a rosette around the centrosphere can be observed in the clasmatoocyte as well as in the monocyte. This is certainly true. It is also well known that at a certain stage in the development of the granulocytes, the neutrophilic, eosinophilic, and basophilic granules are arranged so as to accentuate the centrosphere. This is, however, a transient stage in the development of the leucocyte but this phase will be seen in the blood whenever conditions bring slightly less mature leucocytes from the bone marrow. In studying living cells taken directly from the animal, a rosette formation is the exception for the clasmatoocyte and the rule for the monocyte. Moreover, in the epithelioid cell of the tubercular process, the rosette formation of the neutral red bodies becomes the constant, chief morphological characteristic of the cell.

In an earlier paper in this *Journal* (5), we recorded the finding of clasmatoocytes in the bone marrow filled with acid-fast debris as the epithelioid cells of the local tubercular process degenerated spontaneously. These cells were of the type of the branched adventitial cell of Marchand, stretched along the vessels so that of their identity with the clasmatoocyte or macrophage strain there was no question. In Figs. 6 to 9 is shown the contrast between the reaction of the clasmatoeytic and the monocytic strains to tubercle bacilli as stained by the Ziehl-Neelsen technic. Figs. 6 and 7 are of the monocytic strain. Figs. 8 and 9 are clasmatoocytes. Figs. 6, 7, and 8 are drawings made with the camera lucida of cells from a scraping from the lung of a rabbit (R 216) 4 days after the injection of tubercle bacilli. All three were taken from adjacent fields in the same preparation: Fig. 6 is a young epithelioid cell containing seven intact tubercle bacilli. The cytoplasm stains a deep blue with the methylene blue of the counterstain and is exactly like the reaction of the cytoplasm of the giant cell, Fig. 7. In marked contrast is the pale, foamy, and much vacuolated cytoplasm of the clasmatoocyte, Fig. 8, with one large round nucleus. In the giant cell there are at least three nuclei, and the cell is filled with great numbers of intact tubercle bacilli.

In the two clasmatoocytes, on the other hand, Fig. 8, from the lung, and Fig. 9, from the omentum, of another rabbit of the same series (R 218) killed 8 days after the injection of the bacilli, the reaction is entirely different. The cell of Fig. 9 is the same type as the long clasmatoocyte from the omentum shown on the same plate as Fig. 3. In both Figs. 8 and 9 are shown acid-fast fragments, the cell of Fig. 8 being the key because it has tubercle bacilli apparently in the process of fragmentation. In the omentum from which the cell of Fig. 9 was drawn, it was not simply the occasional clasmatoocyte which showed this reaction, but there were wide zones in which every clasmatoocyte contained the acid-fast debris. Likewise, in the sections for bone marrow which have shown this reaction, it has not been the occasional clasmatoocyte which contained the specific debris, but numerous cells throughout the section.

The occurrence of acid-fast granules in tubercular tissue is well known to the pathologist though it is not regarded as sufficient evidence for a diagnosis of the disease in the absence of, or in the failure to find, the actual intact bacilli, but we are bringing evidence to show that they may be significant in the analysis of how the organism deals with a tubercular infection. Our observations indicate that there is a certain difference in the response of two closely allied strains of mononuclear cells to this specific pathological agent; that, while both clasmatoocytes and monocytes are, under appropriate conditions, highly phagocytic types, the one form tends to break up tubercle bacilli and the other type retains them intact and alive with reproductive multiplication for long periods of time.

In these studies it appears that the immediate major response of the connective tissues to the infection of tuberculosis involves two strains of cells, clasmatoocytes and monocytes. The more chronic response of the lymphocytes is well known through the work of Murphy (18). The clasmatoocytes are the macrophages of the general connective tissues and respond to the presence of large numbers of tubercle bacilli as to any other debris or foreign material, phagocytizing and fragmenting with the attempt to destroy vast numbers of the bacilli. There is evidence that in this process many clasmatoocytes are themselves destroyed. During an infection with tuberculosis

great numbers of clasmatoocytes containing acid-fast debris may be found in the tissues.

With the monocyte, on the other hand, the infection exerts an influence whereby this cell becomes the typical epithelioid type, eventually making the characteristic lesion of the disease; in the epithelioid and giant cells the tubercle bacilli remain intact and apparently survive over long periods of time. The epithelioid cell appears in the lung within 24 hours after the injection of the bacilli, which is correlated perhaps with the observation that in the lung normally at all times are to be found numbers of mature monocytes. In other organs, this normal presence of monocytes is limited, and may represent one of the factors in organ susceptibility; the normal number of monocytes or the degree to which monoblasts may be stimulated to development and maturation, together with the activity of the clasmatoocytes in destroying bacilli, in any particular region would appear to be a function of the rapidity and extent of the local tubercular involvement.

SUMMARY.

1. The early reaction to intravenous tubercular infection in the various organs of the rabbit reveals a pathognomonic response in the lungs within 24 hours; the specific response in the liver, spleen, lymph glands, and bone marrow, follows from the 6th to the 14th days.

2. The development and extent of the pathologic process has been analyzed in terms of the activity of monocytes and clasmatoocytes.

3. The criteria for differentiating these mononuclear phagocytic cells into two strains have been analyzed and the technics discussed.

4. The clasmatoocyte phagocytizes tubercle bacilli freely and fragments them, as it does all cellular and other debris.

5. The monocyte stimulated to metamorphose into the typical epithelioid and giant cell of the Langhans type retains the tubercle bacilli intact, with power to survive and multiply, over long periods of time.

6. The normal number of monocytes or the degree to which monoblasts may be stimulated to development and maturation, together with the activity of the clasmatoocytes in destroying bacilli, in any

particular region, would appear to be a function of the rapidity and extent of the local tubercular involvement.

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EXPLANATION OF PLATE 25.

FIG. 1. Fibroblast from the omentum of a rabbit, R 160, which had received ten intraperitoneal doses of 80 mg. and two of 160 mg. of a phospholipin designated A-3, isolated by Dr. R. J. Anderson from human tubercle bacilli, Strain H 37. The peripheral blood from a similar experiment is shown on Chart 4 (R 153) in a paper in this *Journal* (3). The cells of Figs. 1 to 4 were from the same preparation and were all within the same oil immersion field. Drawn from the living cell stained supravitaly with neutral red. The magnification is shown by the red cell of Fig. 4

FIG. 2. Epithelioid cell from the same preparation as Fig. 1. The color of the reaction of the rosette to neutral red, namely carmine, was carefully matched.

FIG. 3. Resting clasmatoocyte from the same preparation as Figs. 1 and 2. The vacuoles in the preparation when first seen had some red tones of the dye, but were all yellow, as shown, by the time the epithelioid cell of Fig. 2 had been drawn.

FIG. 4. Red cell for the magnification of Figs. 1, 2, 3, and 5.

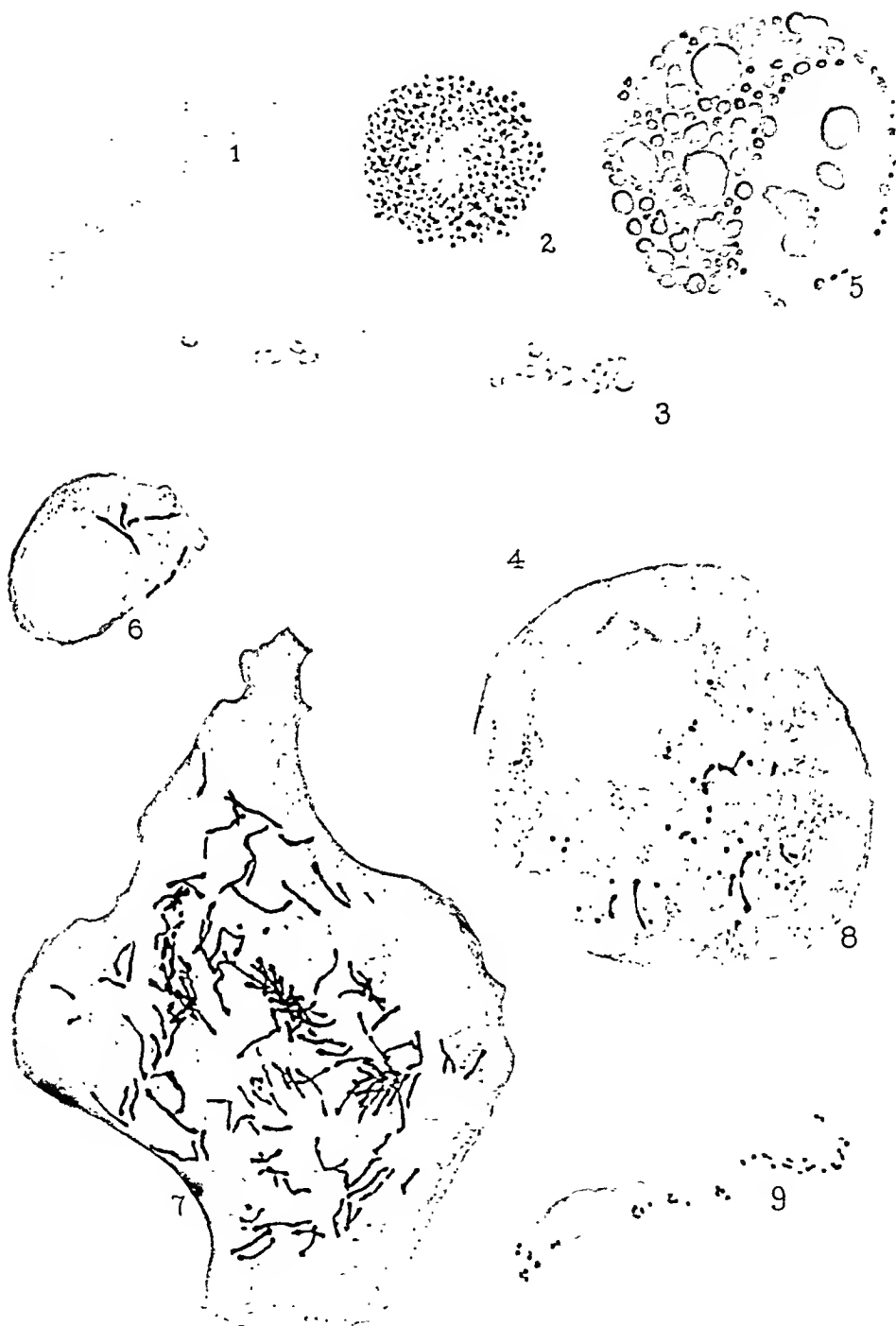
FIG. 5. Clasmatoocyte from the peripheral blood of a rabbit, R 193. The studies of the blood of this animal are shown on Chart 5, in a paper in this *Journal* (5). The cell was drawn on March 22, 1927, 24 hours after the second intravenous injection of bovine tubercle bacilli, when there were 16 per cent clasmatoocytes, 12 per cent degenerating cells, and 8 per cent monocytes in the circulating blood. Drawn from the living cell stained with vital neutral red. The magnification is about the same as that of Figs. 1, 2, and 3.

FIG. 6. Epithelioid cell with seven intact tubercle bacilli from a scraping of the cut surface of the lung of a rabbit, R 216, 4 days after an intravenous injection of 5 mg. of living tubercle bacilli. Stained with Ziehl-Neelsen technic for tubercle bacilli. The cells of Figs. 6, 7, and 8 are all from the same slide; were drawn with the camera lucida, magnification 1700, with the reaction to the methylene blue carefully matched.

FIG. 7. Giant cell of the Langhans type from the lung, showing many intact tubercle bacilli. Drawn from the same slide as the cell of Fig. 6.

FIG. 8. Clasmatoocyte from the lung, showing many tubercle bacilli in the process of fragmentation. From the same preparation as Figs. 6 and 7.

FIG. 9. Clasmatoocyte from the omentum of a rabbit, R 218, 8 days after an intravenous injection of 5 mg. of living tubercle bacilli. Fixed in formalin and stained with Ziehl-Neelsen technic for tubercle bacilli. Drawn with the camera lucida at a magnification of 1700. This cell was one of many in the preparation to show acid-fast debris.



THE BIOLOGICAL REACTIONS IN RABBITS TO THE PROTEIN AND PHOSPHATIDE FRACTIONS FROM THE CHEMICAL ANALYSIS OF HUMAN TUBERCLE BACILLI.

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PLATES 26 TO 29.

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Through the organization of the Research Committee of the National Tuberculosis Association, for cooperative research, we have had available for biological testing certain protein and phosphatide fractions isolated in the chemical analysis of tubercle bacilli. Two specimens of water-soluble proteins, designated 304 and 903 respectively, were received from Professor Treat B. Johnson of the Sterling Chemistry Laboratory of Yale University, and two phosphatide fractions, A-3 and A-4, from Dr. R. J. Anderson, of the same laboratory (1, 2). The material was obtained from the analysis of human tubercle bacilli, strain H 37.

In another paper in this *Journal* (3) evidence has been given to show that clasmotocytes and monocytes react differently toward tubercle bacilli. By some investigators, particularly those using the technic of tissue culture, it is believed that this phagocytic mononuclear group represent essentially a single strain of cells, variations in the physiological environment being thought sufficient to account for differences in the histological structure within the limits observed. However, an increasing weight of evidence accumulating as a result of the direct analysis of the response of the cells of the connective tissues *in situ* to pathological conditions, is necessitating the subdivision of this group on functional, as well as genetical and structural (4, 5) grounds. For example, while both clasmotocytes and monocytes phagocytize tubercle bacilli, the clasmotocyte on the one hand fragments the bacteria readily and rapidly as it does all debris with

which it has to deal; the monocyte, on the other hand, retains the specific bacillus intact so long that the relationship may be considered one of symbiosis.

And now, again, in analyzing the responses of tissue to the protein and phosphatide fractions from the tubercle bacilli, it has been found necessary to express the differential reactions in terms of the same two types of cells within this mononuclear phagocytic group: the response to the protein fractions has been predominantly clasmatocytic, in sharp contrast to the development of monocytes, epithelioid cells, and Langhans giant cells, making the typical lesions of tuberculosis, which is the overwhelming reaction to the phosphatide fractions.

The amount of material available limited the administration of the protein fractions to seven rabbits, the phosphatide fractions to five rabbits, with a control group consisting of six rabbits.

The Protein Fractions.

Both the proteins, 304 and 903, were given intravenously from saturated solutions made up each day in water freshly distilled from glass. From a preliminary experiment (6) it was known that rabbits could readily withstand a dosage of 10 cc. of the saturated aqueous solution of 304. With this experience as a basis a similar dosage of 903 was given to Rabbit R 138, with an immediately fatal result simulating anaphylactic shock. A second rabbit, R 132, survived the initial dose of 10 cc. of 903, but died immediately after a second dose 24 hours later. In the latter animal the white count before the first injection was 9350; on the next day, just before the second inoculation, the white cells had risen to 25,000. At autopsy the lungs showed hemorrhagic foci and the free cells were predominantly clasmatocytes and leucocytes. The protein fraction 903 was, thus, found to be far more toxic than 304, since in the present experiment two rabbits survived ten and eleven doses, respectively, of the latter in the dosage that proved fatal with 903.

To determine the upper limit of sublethal dosage for 304, Rabbit R 152 was given two injections of 20 cc. each at an interval of 24 hours. The studies of the peripheral blood of this animal are shown on Chart 1, the data concerning the white cells being given on a scale one-half the magnitude of the other graphs to cover the rise in leucocytes. The dosage of 20 cc., while not lethal, was too toxic to study other than the acute reaction; the day after the first injection the animal appeared ill, ate but little, and showed a rectal temperature of 107.6°F. (normal 102–104°F.); following the second dose the condition did not warrant further injections. From the chart it is obvious that the animal was developing an anemia, the red cells

falling from 6,800,000 to 4,100,000 and the hemoglobin from 65 to 53 per cent; while the white cells mounted from 10,000 to 27,000. On the two counts made before the injection, the neutrophilic leucocytes were low, 38 and 29 per cent, as can be seen on the chart by a comparison of their number and the total number of the white cells. However, the total myeloid cells were about half the white cells, 54 and 57 per cent, due to the relatively large percentage of basophilic cells, which were 13 and 22 per cent in respective counts. 24 hours after the first injection a marked rise in the total number of white cells may be seen to have been due wholly to an increase in neutrophilic leucocytes and this continued to be true throughout the experiment. The basophilic and eosinophilic leucocytes, on the other hand, remained unaffected at their original levels indicat-

ing a specific stimulating effect on one group of granulocytes only, namely the neutrophils. There were interesting qualitative as well as quantitative changes in the neutrophilic leucocytes: on Jan. 4, of the 67 per cent neutrophils, 57 per cent were of the non-motile (7) type. These are the forms that appear as ruptured cells with scattered granules in fixed films, showing that they are fragile. 24 hours after a second dose of 304 was given, the white cells showed some further increase but now there were found two types of neutrophils, old, degenerating types with fragmenting nuclei and a new group of peculiar young forms. These young leucocytes had indented nuclei, not yet two lobed; they were markedly deficient in the specific granulation and rich in mitochondria; and they were not actively amoeboid. In fixed films these cells had the other characteristic of incomplete maturation, namely a strongly basophilic cytoplasm. At this examination the leucocytes were about equally divided between the old forms and the young. A second count, in the afternoon, showed a fall in total number of white cells to within normal limits with

only 6 per cent of the degenerating leucocytes remaining. On the next day the total white count had risen again to 27,000 and the young leucocytes as described were the predominating type. As will be seen on the chart, both monocytes and clasmatocytes rose slightly while the lymphocytes remained at the low level to which they had fallen after the first dose.

The studies of bone marrow following the autopsy on the 4th day revealed something of the nature of the processes already indicated in the studies of the peripheral blood. The bone marrow had been depleted rapidly of its store of mature myelocytes, Type C (8): this was evident in the supravital preparations of the fresh marrow and in sections. The sections showed that the interspaces

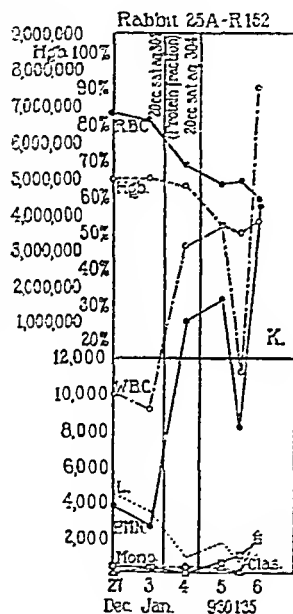


CHART 1.

between the fat cells had been denuded of cells so that they were like the sections of marrow shown by Doan *et al.* (9) both from experimental animals treated with repeated doses of dead typhoid bacilli and from a human case of typhoid fever. In the supravital preparations of this marrow the immature myelocytes, Type B, with fewer of the specific granulations, showed a premature indication of polymorphism of the nuclei and the beginning of motility. Thus the leucocytes of the blood stream had had certain of the characteristics of the B myelocytes, namely the basophilic cytoplasm, the large content of mitochondria, and the low content of specific granules; while their nuclei were, on the other hand, of the type of the metamyelocyte, that is indented but not yet lobed. The nuclei showed that the cells were being transformed into functionally active leucocytes without complete maturation relative to their full quota of granules. These cells therefore were entirely different from the immature and unchanged B myelocytes that occur in the blood stream in leucemia (8). This type of cell, the immature but motile leucocyte, is especially interesting because it occurs in the blood in typhoid fever (10), probably for the same reason as in this rabbit with the massive doses of protein. It must be discriminated with care from the monocyte both in the fixed films and in the supravital technic. The discrimination is to be made by the nature of the granulations. In this rabbit, it is interesting to note that the effect of the protein was limited to the neutrophilic (pseudo-eosinophilic) leucocytes, both the basophilic and the eosinophilic cells being unchanged in the blood and in the bone marrow; the reaction thus represents a response to a protein in one strain of granulocytes. This type of reaction is of course not specific of a protein from tubercle bacilli only.

The autopsy showed a general increase in clasmatocytes in the tissues in the lung; as determined in supravital studies, many of these clasmatocytes contained fragments of red cells. In sections the interalveolar septa were thickened and a few foreign body giant cells were found. As in all the animals with the protein fractions, there were hemorrhages. In this animal they were especially marked in the bone marrow as shown by sections. There were gross hemorrhages in the retroperitoneal tissues extending into the psoas muscle on either side.

The liver showed changes. There were certain chronic periportal lesions entirely independent of the experiment. The acute lesions of the liver were irregular, small zones of necrosis, involving 20 to 30 liver cells, sometimes centrally placed, but more often in the middle of the lobule part way between a portal space and a central vein. In these zones the liver cells were extremely vacuolated with no evidence of normal granulation and the nuclei showed extensive fragmentation. Frozen sections stained with Sudan III showed these cells loaded with fat. Leucocytes and highly phagocytic clasmatocytes infiltrated these areas, with destruction so great that the vessels could not be analyzed. There were also degenerations in the convoluted tubules of the kidney, some of which contained cellular debris and some fragments of red cells. Thus the effects of the protein in this animal were a specific leucocytosis, fever, an increase in clasmatocytes in the lung, multiple hemorrhages, focal fatty necrosis of the liver cells, and some damage to the cells of the kidney tubules.

TABLE I.

Protocols.

No. of rabbit	Dose	Length of life
All protein fractions given intravenously		
R 138	1 dose of 10 cc. of 903	Died immediately after first dose
R 132	2 doses of 10 cc. of 903	Died after second dose
R 152	2 doses of 20 cc. of 304	Killed 48 hrs. after second dose. Maximum temperature 107.6°
R 131	26 doses of 2 to 5 cc. of 903. Total of 72 cc. in 33 days	Killed 3 days after last injection. Maximum temperature 107°
R 137	25 doses of 1 to 3 cc. of 903. Total of 32 cc. in 31 days	Died immediately after last injection; complicating peritonitis found, due to <i>B. lepi-septicum</i> . Maximum temperature 108.2°
R 129	13 doses of 6 to 10 cc. of 304. Total of 126 cc. in 15 days	Killed 24 hrs. after last dose. Maximum temperature 108.5°
R 130	12 doses of 6 to 10 cc. of 304. Total of 116 cc. in 15 days	Died night after last dose. Maximum temperature 108.3°
Phosphatide fractions		
R 156	2 doses of 80 mg. of A-3 intravenously	Acute death suddenly after second dose, from emboli
R 153	2 intravenous and 12 intraperitoneal doses of from 80 to 160 mg. of A-3. Total of 1280 mg. in 15 days	Killed 24 hrs. after last dose
R 160	12 intraperitoneal doses of from 80 to 160 mg. of A-3. Total of 1120 mg. in 13 days	Killed 48 hrs. after the last dose
R 158	1 intravenous and 12 intraperitoneal doses of from 40 to 160 mg. of A-4. Total of 1000 mg. in 14 days	Killed 6 days after the last dose
R 159	1 intravenous and 12 intraperitoneal doses of from 40 to 160 mg. of A-4. Total of 1120 mg. in 14 days	Killed 4 days after the last dose
Controls		
R 243	15 intraperitoneal doses of from 80 to 122 mg. of lecithin. Total of 1242 mg. in 17 days	Killed in excellent condition 24 hrs. following the last injection
R 244	15 intraperitoneal doses of from 80 to 122 mg. of lecithin. Total of 1242 mg. in 17 days	Killed in excellent condition 24 hrs. following the last injection

TABLE I—*Concluded.*

No. of rabbit	Dose	Length of life
Controls— <i>Concluded</i>		
R 245	15 intraperitoneal doses of lecithin plus tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 1242 mg. lecithin and 3.75 mg. bacilli in 17 days	Killed in excellent condition 24 hrs. following last dose
R 246	15 intraperitoneal doses of lecithin plus tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 1242 mg. lecithin and 4.00 mg. bacilli in 17 days	Killed in excellent condition 48 hrs. following last injection
R 247	12 intraperitoneal doses of tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 3 mg. in 14 days	Killed in excellent condition immediately after last injection
R 248	16 intraperitoneal doses of from $\frac{1}{4}$ to 3 mg. of tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 7 mg. in 19 days	Killed in excellent condition 24 hrs. after last injection

With the studies on toxicity just cited as a basis for dosage the remaining material was given to a small number of animals, rather than less amounts to a larger series in the hope of securing if possible a striking and characteristic effect. The results have seemed to justify this plan. Two rabbits (R 131 and R 137) received the protein 903 in repeated daily doses of 1 to 2 cc. of a saturated solution, with final doses of from 3 to 5 cc.; while two others (R 129, R 130) were given daily intravenous injections of 304 in the original amounts of 10 cc. per dose. With these dosages in total amounts as shown in Table I, the effects noted in the four animals were in general comparable.

Chart 2 is given as representative of the two animals with 903, Chart 3 for the two with 304. These charts are given for the sake of comparison of the effects on the peripheral blood of the chemical fractions from tubercle bacilli with the known effects in the rabbit of the living tubercle bacilli in large doses. In an earlier paper in this *Journal* (11) it has been shown that the changes in the blood after an intravenous injection of 1 or 2 mg. of bovine tubercle bacilli reflect directly, and may be interpreted in terms of, two different processes. First, an anemia combined with the fall in platelets and granulocytes indicates a direct effect on the bone marrow; second, an increase in monocytes and at times in clasmotocytes combined with a fall in lymphocytes is correlated with the general progress of the tubercular process in the tissues (6).

It has been demonstrated that the effect revealed in the blood in red cells, plate-

lets, and granulocytes is due to an extensive involvement of the marrow with tuberculosis, a constant finding at a certain stage with the doses mentioned; that the local tubercular lesions are sufficient to displace the fat and to reduce the marrow to the level of the early erythroblasts for the red cells and the early myelocytes for the white cells. Rabbit R 131 received 903, in doses as seen in Chart 2. It will be seen in this chart that there is a sharp fall of the red cells, correlated with a drop in hemoglobin beginning after the 8th dose of protein; the lowest point was reached Dec. 30 with 3,760,000 red cells. There was later a partial recovery of the red cells, but to an average of 5,000,000 rather than to the original average of 6,000,000. This fall in red cells was not correlated with a fall in granulocytes,

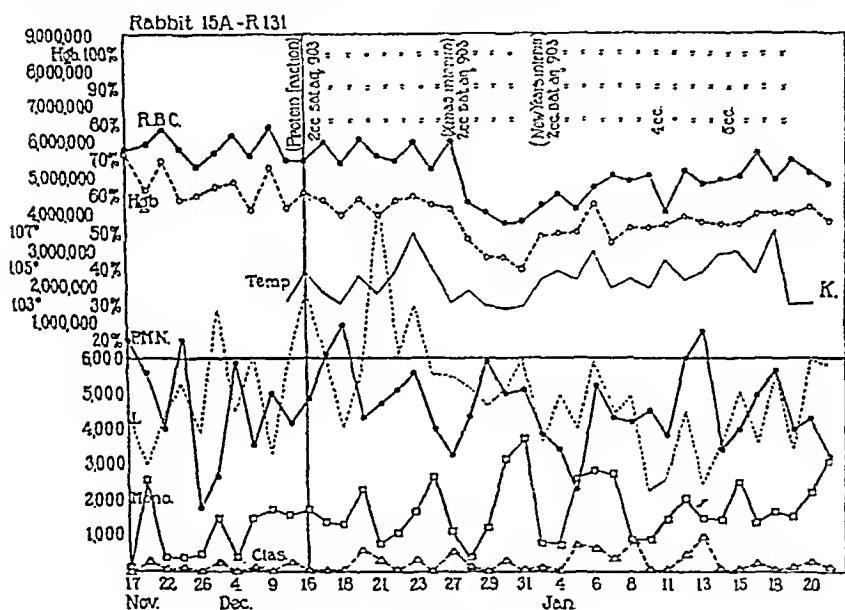


CHART 2.

the latter remaining practically at their original level. The anemia was found to be due to multiple hemorrhages and was not therefore a local effect on bone marrow except in so far as there were, as in every case, hemorrhages in the bone marrow itself as well as multiple ones outside the marrow. The anemia was thus secondary to the hemorrhage.

In regard to the white cells, the total numbers are not shown on the chart but their range before the injections was from 7250 to 15,600; while after the injections there were four moderately high counts: Dec. 21, 17,000; Dec. 23, 15,600; Dec. 31, 15,400; and Jan. 6, 15,900. These numbers are not excessive for the rabbit and the experiments already recorded with R 132 and R 152 indicate that a marked

effect of the protein on the leucocytes is obtained only with larger doses. The chart also differs from a typical tubercular chart in a negative effect on lymphocytes. On the other hand, there was a somewhat rhythmic rise in monocytes and clasmatocytes which is like tuberculosis. At the times of the increase in monocytes in the peripheral blood there were some qualitative changes, namely some decreased motility, an increase in small scattered neutral red bodies in the cells, and some cells with accentuated rosettes; but no true epithelioid cells were found such as had been seen in the preliminary tests with 304 (6).

Rabbit R 137 received smaller doses of 903, namely, twenty of 1 cc., three of 2 cc., and two of 3 cc., making a total of 32 cc. as contrasted with a total of 72 cc. for R 131. R 137 had the only complication of the series, namely a terminal peritonitis due to the *B. leipisepticum*, identified by Dr. Ida Pritchett. The only difference between this chart and that of R 131, as far as the white cells were concerned, was a fall in both leucocytes and lymphocytes during the last 4 days. In this animal there was no anemia. The autopsy showed only small fresh hemorrhages so the interpretation is that the animal did not survive the hemorrhages long enough to show their effect in the peripheral blood. The curves of monocytes and clasmatocytes were the same as in the corresponding rabbit, R 131.

The peripheral blood in the two animals, R 129 and R 130 (Chart 3), that received the protein fraction 304, showed the same effects as 903. R 129 received eleven doses of 10 cc. and one of 6 cc. Rabbit R 130 had ten doses of 10 cc. and one of 6 cc. Both animals showed a gradually progressive anemia; the lowest count on R 129 was 3,680,000 from an original base line of 5,500,000; while the lowest count on R 130 was 2,970,000 from a level of 5,000,000 to 6,000,000 before the injections. Of all the animals with the protein fractions, R 130 showed the most marked anemia and had the most extensive hemorrhages. In both R 129 and R 130 there was a neutrophilia and a tendency toward lower lymphocytes. In R 130, though not in R 129, there was the same somewhat rhythmic rise in monocytes as is shown on Chart 2.

In summary, the protein fractions 903 and 304 were both toxic; both produced anemia with neutrophilia of greater or less degree; four of the rabbits died, two (R 138 and R 132) in the acute reaction to large doses of 903, one (R 130) after repeated doses of 304, and the other (R 137) after 903 but with a complicating infection so that death could not be ascribed to the protein alone. There were no signs of secondary infection in the other animals. One rabbit (R 152) was killed when in a critical condition; the other two (R 129, R 131) were killed while still in good condition. All the rabbits appeared more or less ill with temperatures reaching between 107° and 108.5°.

At autopsy the findings in the series were quite uniform. The only signs of abnormality obvious in the gross inspection were multiple hemorrhages. In every animal, on opening the abdominal cavity, there was found edema of the prevertebral connective tissues, with small hemorrhagic foci scattered throughout from diaphragm to pelvis. These hemorrhages extended characteristically into the septa of the psoas muscles on either side and in one animal were found to have

dissected into the prevertebral tissues of the thoracic cavity and were found also in the axillæ. The question arose as to whether the psoas hemorrhages, so uniform in distribution in the gross, could be traumatic in origin, but they were present in only these five out of more than seventy rabbits handled in the same way by the same people; moreover, all the rabbits in this group had also hemorrhages in the bone marrow as well as in some of the other organs. The animal with the infection (R 137) had a peritonitis with a thick, purulent exudate covering the entire peritoneal surfaces, but typical hemorrhages were found in sections of the subperitoneal prevertebral tissues.

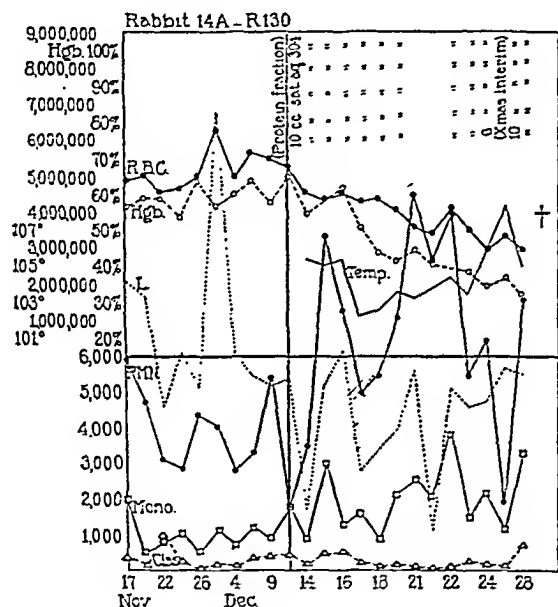


CHART 3.

In Fig. 6, is shown a drawing from the central part of the mesenteric lymph gland of Rabbit R 131, which drained a hemorrhagic area. The lymph cords are covered, or, in other words, the lymphatic sinuses are lined by endothelial cells which are engorged with debris from red cells. In this lymph gland the peripheral sinuses were entirely denuded of endothelium leaving quite bare the scanty framework of reticular cells which showed no phagocytic activity whatever. All the central sinuses of the gland show a complete phagocytic endothelium except for an occasional endothelial cell which has dropped off as is indicated by the arrow *b* (Fig. 6). At the point marked with the arrow *a* on the drawing there is a reduplication of the endothelium, with the outer cell rounding up as if to des-

quamate. At the point marked *c*, there is an endothelial cell stretching along the reticular framework. Within the lymph cords there are a few phagocytic cells exactly like the lymphatic endothelium; some of these are obviously adventitial cells to the blood vessels of the lymph cords; others appear to be free of any relation to the adventitia of a blood vessel. There was no involvement of the blood vascular endothelium *per se* in the phagocytic activity. In some of the central sinuses there were great numbers of free round phagocytes loaded with the debris, which cells probably account for the endothelium of the denuded peripheral sinuses. This lymph gland offers as good evidence as can be had that certainly some of the cells of the free phagocytic type, the typical clasmatocyte, may originate from endothelium, in this case from lymphatic endothelium. It also gives quite conclusive evidence that there are two types of cells in the lymphatic sinuses, the non-phagocytic reticulum and the potentially active endothelium; and it shows as well that the endothelium is complete or incomplete according to the functional state of the gland. It is probably true that the extensive response of the endothelium in this lymph gland was not a primary effect of the protein injected but was rather a response secondary to the presence of a massive amount of the debris of red cells. In this rabbit there was one rather large hemorrhage in the edge of the psoas muscle near the kidney and there are clumps of clasmatocytes loaded with yellow debris and others with brown pigment from the red cells in the surrounding tissues. This is the only rabbit in the series in which there has been any breaking down of the hemoglobin in any of the hemorrhagic areas sectioned. On Chart 2 from this animal (R 131), it will be seen that there was a sudden marked fall in the red cells, 24 days before the date of the autopsy; whereas on Chart 3, the fall in the red cells was a very gradual one. All the other hemorrhagic areas studied in the other animals show the condition illustrated in Fig. 9, in which there are intact free red cells in the tissues exactly like those in the vessels without any appreciable phagocytosis. Obviously there has been a marked increase in connective tissue cells, with many mitoses present. In the supravital studies of the tissues adjacent to that of Fig. 9, the predominating cell was identified as the clasmatocyte in its characteristic reaction to neutral red but no phagocytosis of the red cells was found. The interpretation is that these areas show a preliminary increase in the clasmatocytes by division before the red cells are engorged. In the fixed tissue, as shown in Fig. 9, it is clearly not possible to discriminate between fibroblasts and clasmatocytes; and it is well known that the inactive clasmatocyte has never been discriminated in fixed tissues. With the supravital technic as applied to the connective tissues, however, it is possible to distinguish readily the cell types morphologically differentiated and known as the fibroblast, the resting clasmatocyte, the stimulated clasmatocyte, the epithelioid cell, and the serosal lining cell (see plates, etc., in preceding papers (4, 6)).

Beside the hemorrhages, all the rabbits with the protein fractions showed a thickening of the interalveolar septa in the lungs. The maximum grade of this lesion found in any of the series is shown in Fig. 7. In the gross specimens the lungs did not look abnormal, but there was an increased resistance to the knife on cutting

and the supravital preparations all showed an increase in free cells which were of the clasmotocytic type. In all the lungs the areas of thickened septa were in patches and varied in amount from a lesion just perceptible up to the grade shown in Fig. 7. This section is from a rabbit, R 130, which was found dead. The lungs were mottled, which was postmortem, but supravital preparations were recorded as showing a marked increase in free cells, entirely due to clasmotocytes which were still living as shown in their characteristic reaction to neutral red. It is interesting to note that this type of reaction has already been found in response to the injection of both living and dead tubercle bacilli. It is this reaction which Lewis and Sanderson (30) found in the lungs 48 hours after the injection of massive doses of living bacilli, demonstrated to be an increase in clasmotocytes in another paper in this *Journal* (3). The reaction in the lung to the injection of dead bacilli or of chloroform extracts has been described in the literature (14, 18, 31) as an interstitial pneumonia, which is the lesion illustrated in Fig. 7.

In describing the autopsy results in Rabbit R 152, with massive doses of 304, it was noted that there were small necrotic areas in the liver. There was another rabbit in the series, R 130, with extensive acute necrosis of the liver cells. At autopsy, the hepatic lesions were easily visible in the gross, giving a marked mottling to the surface; frozen sections showed areas of cells loaded with fat and in fixed sections these zones showed extreme necrosis of the liver cells with destruction of their nuclei. The lesions are the same as in Rabbit R 152 but much more extensive. These are acute lesions, having no relation to the chronic periportal cirrhosis found in all the rabbits of the series. Inasmuch as the acute necrosis of liver cells was obvious in only two of the five animals, it cannot be regarded as a constant effect of the protein; it must, however, be kept in mind in the further biological testing of the material.

The lesions found in all the animals that received the protein fractions were multiple hemorrhages from small vessels and an increase in clasmotocytes, especially in the so called interstitial pneumonia of the lungs. These proteins were thus toxic to endothelium. In general, the proteins were toxic to the animal, lethal in large doses, and gave high temperatures.

Phosphatide Fractions.

Known weights of the phosphatide fractions were rubbed into a fine emulsion in water freshly distilled from glass. The dose determined with reference to the proportion of this material to the total weight of the dry bacilli was 80 mg. The first injections were made intravenously but since one of the animals dropped dead instantly, probably from an embolus, the intraperitoneal route was substituted.

Four rabbits were given repeated intraperitoneal doses of the two fractions, as shown in Charts 4 and 5. Two rabbits, R 153 and R 160, received A-3, while two rabbits, R 158 and R 159, had A-4. These two fractions were entirely non-toxic and there was no rise in temperature in any animal. The two rabbits that received A-3, of which Chart 4 is representative, both showed an anemia with a

fall of about 2 million red cells and a corresponding drop in the hemoglobin. At the same time both of them showed a rise in monocytes reaching the level of 4000 cells per c.mm. The two rabbits that received the fraction A-4, of which Chart 5 is representative, showed the same response in less degree. In these animals, R 158 and R 159, the fall in red corpuscles was a little over a million cells while the rise in monocytes was to the level of 2000 cells. The changes in leucocytes and lymphocytes were inconstant and perhaps negligible; Rabbits R 153 (A-3) and R 159 (A-4) both showed the slight drop in leucocytes and lymphocytes indicated on Chart 4, while the other two had no changes, as shown on Chart 5. Thus all

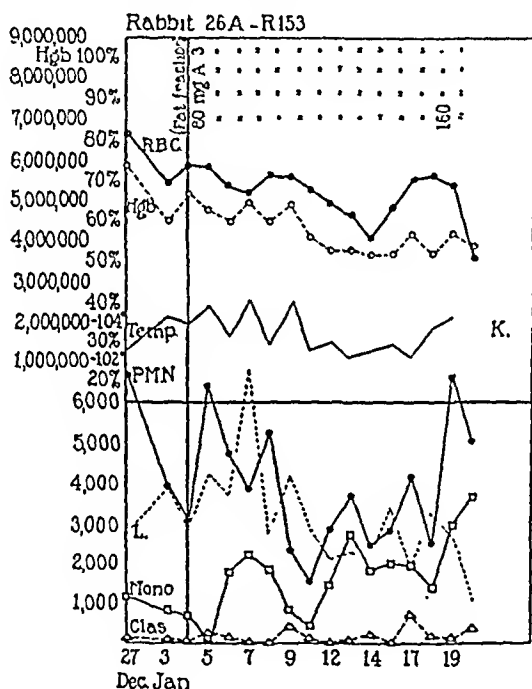


CHART 4.

four animals showed some anemia, which was possibly due to the distilled water introduced, since there were no hemorrhages and no changes were detected in the bone marrow. In all four animals there was a rise in monocytes, after A-4 to 2000 cells, after A-3 to 4000 cells. Thus the greater anemia and the more marked monocytosis were with the fraction A-3.

The four animals with the phosphatide fractions, in striking contrast to those receiving the protein, showed no clinical symptoms whatever during the course of the injections. The rabbits, therefore, were autopsied while all were yet in excellent condition when all the available material had been used. The extent of the pathological lesions was thus entirely unexpected. The findings in general

were identical in type in the four animals and the pathology in general was confined essentially to the peritoneal cavity. The local lesions were in every case so massive that it was difficult to differentiate quantitatively their relative extent in the individual cases. Rabbit R 128, which received A-4, had perhaps the greatest amount of involvement though it was one of the rabbits with the lesser reaction in the peripheral blood.

On opening the peritoneal cavity there were no ascites and no adhesions but all the animals showed an extensive involvement of the parietal and visceral peritoneum with what proved to be typical tubercular tissue. The omentum in every case

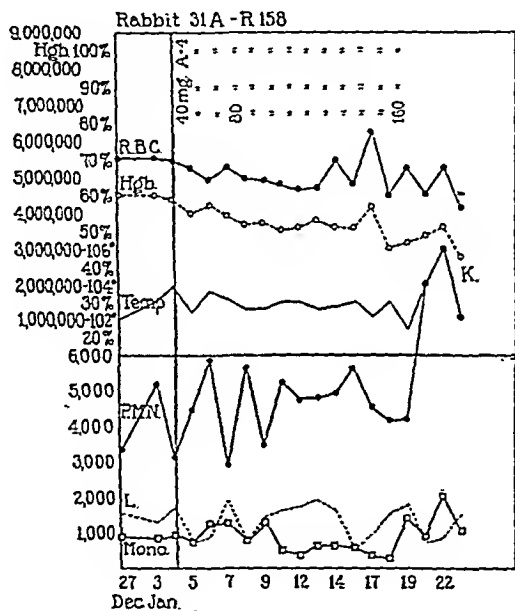


CHART 5.

was a dense thickened, nodular, dull reddish mass, markedly different from the delicate membrane of the normal state. The characteristic color was probably due to the increased vascularization. Some idea of the thickness of these omenta may be obtained from Fig. 11, in which the original thickness of the omentum illustrated can be seen at the upper right hand corner; the rest of the photograph gives about one-third of the actual thickness of the tissue at that point. The proliferation is specifically of epithelioid cells with a great excess of giant cells of the Langhans type in this area. The small darker nuclei indicate clusters of lymphocytes. Fig. 10 illustrates another area in the same animal (R 153) with fewer giant cells and a marked proliferation of typical epithelioid cells. The

predominating cell from the omentum of this animal is shown in the supravital technic as Fig. 5. This epithelioid cell is to be compared with a similar one, Fig. 3, which was found in the peripheral blood of a rabbit, R 19, 16 days after the injection of 1 mg. of living bovine tubercle bacilli. The graph showing the peripheral blood of this animal is given as Chart 1 in another paper in this *Journal* (11), and the cell was drawn on Dec. 2 when there were 6000 monocytes per c.mm. in the peripheral blood. In both of these epithelioid cells is shown the typical rosette of tiny bodies, with the carmine reaction to supravital neutral red, which is the characteristic of the cell that makes the tubercle. In contrast to these epithelioid cells (Figs. 3 and 5) which far outnumbered any other type of cell in the lesion, are three other cells illustrated from the same omentum as the cell of Fig. 5. Fig. 1 was an unstimulated fibroblast; entirely typical of the type. The cells of Figs. 2 and 4 were in our interpretation somewhat stimulated, rounded up clasmotocytes. They are to be compared with the unstimulated clasmotocyte of the omentum shown in Fig. 3 from an accompanying paper in this *Journal* (3). This cell was from the omentum of another of the rabbits, R 160, that received the phosphatide fraction A-3, and was identical with many found in R 153. But beside these long forms there were also found cells, such as the ones shown in Figs. 2 and 4. In our interpretation these latter cells were types to be classified as clasmotocytes. The cell of Fig. 2 is the more typical; the cell of Fig. 4 showed the tendency of the smaller vacuoles to fading toward yellow; the vacuoles when originally seen were a deeper red than is shown in the drawing, but they were ultimately orange and yellow in color while all the epithelioid cells in the preparations still showed an unchanged carmine reaction. Reference to the photograph, Fig. 10 (R 153), from the connective tissues near the bladder, shows the predominating cell to be the epithelioid type, with eccentric nucleus, but there is an occasional atypical cell with the nucleus in the center as in Figs. 2 and 4. Proof will be given in the discussion of the controls that the clasmotocyte as well as the monocyte takes in lipoids. However, the contrast in the reaction of the cells under discussion in an environment as identical as there would be within the area of omentum included in one oil immersion field of the microscope is strikingly shown in Figs. 2, 4, and 5, or in Figs. 2 and 3 in the accompanying paper in this *Journal* (3).

These are the observations. Concerning them, two different interpretations are possible; either the same type of cell is responding in two different ways, thereby making it necessary to postulate differences in the environment; or, two potentially different strains of phagocytes are reacting differently in the same environment in their disposal of phagocytized material. Our interpretation at the moment favors the latter explanation as the more probable.

In all four of the phosphatide animals there was marked involvement of the parietal peritoneum, especially near the site of injections. The visceral peritoneum covering the pelvic organs and the mesentery were extensively involved while the surface of the intestine had everywhere patches of the same tubercular tissue that characterized the omentum. Most of the lesions were nodular, white

or of the same dull reddish color as the omentum. In Fig. 8 is shown a section of the duodenum at the site of involvement in Rabbit R 159. It shows that the lesions are here limited to the serosal coat. The magnification is low to show the extent of the new growth. The mass of the tissue is of epithelioid cells. The dark patches in the center are lymphocytes; the two dark areas on the left show necrosis with infiltration of leucocytes. The zone in the upper left corner is the more markedly necrotic. In one of the animals there was some involvement of the submucosa, probably by extension. In this same animal (R 159) there was an occasional small tubercle in the liver and a few epithelioid cells in the lung, but in general the reaction was almost entirely localized to the peritoneum. In one animal only, R 158, was there an involvement of the mesenteric lymph glands, as shown in Fig. 12. Here the peripheral follicles are seen extensively involved with typical epithelioid cells, giving a picture that would be described as early tuberculous involvement, though no acid-fast bacilli could be found.

Complete parallel surveys of all tissues from all animals receiving either the protein or phosphatide fractions have yielded uniformly negative results with the Ziehl-Neelsen technic. The phosphatide material itself gave a diffuse reddish reaction with the carbolfuchsin and some of the large giant cells, such as are shown in Fig. 11, stained a faint, diffuse pink. In the case of the lymph gland of Fig. 12 there were certain areas within the follicles and in the reticulum of the lymphatic sinuses in which groups of cells were found containing acid-fast debris. There were, however, no intact, acid-fast bacilli found in any instance.

The contrast between the reaction of the connective tissues to the protein fractions on the one hand and to the phosphatide material on the other may be seen at a glance in comparing the tissues of the lung, as shown in Fig. 7, with the omentum of Fig. 11. In Fig. 7, the reaction is not at all that of tuberculosis. The contrast is, however, much clearer in Figs. 9 and 10, where the tissues involved are entirely comparable. Fig. 9 is from the prevertebral tissues near the kidney after the protein, and Fig. 10 from the connective tissue near the bladder after the phosphatide fraction. The character of the round cells with eccentric nuclei, together with the typical giant cells of a tubercular lesion in the latter, contrast markedly with the long and the branched cells with no trace of a tubercular reaction in the former. In both of these photographs there is shown a little perivascular reaction. In none of the tissues in the entire series was any tendency toward fibrosis seen. In the tubercular lesions after the phosphatide fractions there was a considerable new growth of vessels.

These are the preliminary tests of substances from the chemical analysis of the tubercle bacillus. Under the plans of the Research Committee of the National Tuberculosis Association, Dr. William Charles White, chairman, these tests are to be extended, both to the products from the further analysis of these protein and phosphatide fractions and to comparable substances from other organisms.

As far as these studies have progressed, it is clear that certain of the responses to the proteins are like those to other proteins; for example, the anaphylactoid response to large doses, and the direct effect on the neutrophilic leucocytes from an excessive dose, and possibly the temperature. The acute effect on the liver cells in two animals can only be evaluated with a larger series of animals. With the proteins, all the rabbits showed fever, multiple hemorrhages, and a tendency toward an increase in the cellular response of clasmato-cytes. How far the toxic effect on endothelium and the pressor effect on clasmatocytes are specific awaits further tests. The phosphatide fractions showed only one response, namely the local production of the typical lesion of tuberculosis. There was a massive local increase in monocytes, epithelioid cells, and Langhans giant cells; there was some infiltration of this tubercular tissue with lymphocytes and there was some necrosis. Thus, with these two types of substances from the analysis of the bacilli many of the effects of the actual disease have been reproduced. The typical distribution of the lesions of the infection with living bacilli was not reproduced. If the observations that in the disease itself clasmatocytes fragment the living bacilli, that the clasmatocytes are in turn disintegrated, and the resulting debris rephagocytized, actually mean that large numbers of bacilli are killed and broken down by the cells of the animal, then the actual progress of a tubercular infection involves the effects of both living and dead bacilli on the body.

Controls.

Before venturing any interpretation as to the specificity of the above reactions of the fractions from tubercle bacilli, adequate controls are, of course, essential. It will be possible to secure soon various bacterial proteins with which to compare and contrast the reactions already noted with the protein fraction from the tubercle bacilli. In some respects the acute reactions with this material have approximated anaphylactoid phenomena; in other respects the responses have been unlike the non-specific protein reactions with which the literature deals. Therefore, the real significance of these observations awaits further studies.

As a partial control for the findings with the specific phospholipins of the tubercle bacilli, certain appropriate, if inadequate, experiments were carried out. When stained by the Ziehl-Neelsen technic, it was found that this material, as received, contained a limited number of intact, acid-fast bacilli, the protein fractions being free of such findings. However, when the protein and phosphatide fractions were injected into guinea pigs directly, or inoculated on Petroff's media,

or when the tissue suspensions from rabbits, treated as described above with these materials, were inoculated into guinea pigs, there was no evidence either from repeated tuberculin tests or at autopsy, that any of the bacilli were viable. Ten guinea pigs were used; five were autopsied 2 months after inoculation with completely negative findings, and five are still living and in perfect health 7 months after. Therefore, the control series of rabbits was divided into three groups: one to receive lecithin (12)¹ alone in doses comparable to that given in the case of the phospholipin; another to receive the same dosage of lecithin plus a known added weight of dead human tubercle bacilli, Strain H 37; the third to receive dead bacilli alone, all conditions being identical with those existing during the phosphatide series.

It has long been known that the injection of dead tubercle bacilli can produce lesions when injected into the living animal (13, 14). Koch (15) himself found that they caused aseptic pus upon subcutaneous inoculation. That the lesions produced with the dead organisms may be tubercles with typical epithelioid and giant cells is also well recognized (14, 16-20). Typical of this early work is that of Prudden and Hodenpyl (21) who in 1891 injected rather large amounts of dead bacilli, 2 to 3 cc. of a milky suspension, by various routes, subcutaneous, intrapleural, intraperitoneal, and intravenous. With the intrapleural and the intraperitoneal routes, two animals, one in each series, developed a few small nodules of epithelioid cells in the serosa. With the intravenous route, the tubercular tissue was confined to the lungs. Prudden (22) then studied the lungs after intratracheal injections, in which he also found some reaction in the pulmonary tissues.

In the attempts to explain the mechanism of the reactions noted, the non-specific foreign body stimulus of the bacteria as such is mentioned (18, 23), but it is evident that the preponderant opinion of investigators has been that the histological lesion caused by the tubercle bacillus is due to a poison liberated from the body of the bacillus by the action of the tissue cells (13, 14, 17, 23, 24, 25). Among the first to attempt an analysis of the effects of chemically separate constituents of the dead tubercle bacilli was Weyl (26). He obtained two substances from the bacillus, one of indifferent nature with the tinctorial properties of the bacteria, and the other a toxomucin which, upon subcutaneous injection, caused dry necrosis of the skin. Auclair (27) reported the production of caseation necrosis in guinea pigs by subcutaneous and intratracheal injections of lipoidal materials from human tubercle bacilli extracted by ether, chloroform, xylene, and benzene. In 1903 he studied the tissue reactions following ether extracts of typhoid, Friedländer's, and Loeffler's bacilli, streptococcus, *Staphylococcus aureus*, gonococcus, and actinomyces, finding in contrast to the effects of extracts of tubercle bacilli no typical epithelioid proliferations but rather in each instance only inflammatory lesions comparable to those resulting from the original organism in its usual manifestations. Morse and Stott (23) produced typical cellular tubercles, without caseation, in rabbits and white rats, by subcutaneous intraperitoneal, and intravenous injections of the alcohol extract, but found the

¹We are indebted to Dr. P. A. Levene for the lecithin used in the control animals.

ether extract to be inert. They attributed the characteristic microscopic lesion to waxy substances acting as a peculiar type of foreign body. Gaehlinger and Tilmant (28) found non-specific "liver lipoids," when injected subcutaneously, capable of producing tubercles with caseation, though Ray and Shipman (29) could not confirm this observation. The latter investigators found chloroform-soluble lipins extracted from tubercle, grass, and colon bacilli to produce "entirely similar epithelioid tubercles" when injected *via* the subcutaneous and intrapulmonary routes in guinea pigs. The differences in the degree of the involvement were not considered significant, the conclusion being drawn that the characteristic histological features of tubercles are "merely a foreign body reaction." However, their controls of sodium stearate emulsion, with and without 0.5 per cent chloroform, and olive oil, with and without 0.5 per cent chloroform, gave no lesions. True caseation was never seen by them and they question previous findings of this nature because of the close macroscopic resemblance of the solid pus of rabbit and guinea pig to caseous material. Thus it will be seen that the literature provides to some degree controls for the work presented in this investigation. However, the chemistry of bacteria is being placed upon a new foundation today with the improved methods of quantity production of known strains of organisms on synthetic media of known composition, and with the development of chemical procedures yielding material in amounts sufficient both for analysis chemically and biologically. Also the newer criteria for cell identifications are making the study of the biological reactions more specific and exact than was possible in the earlier investigations.

Charts 6, 7, and 8 are representative of the peripheral blood, respectively, in the three control groups here cited. No changes whatever are revealed in any of the graphs representative of temperature, weight, red cells, hemoglobin, or white cells of the various strains, in the periods of experimentation following the control periods. The fluctuations noted are quite within the range of normal for each determination. Thus, the clinical and peripheral blood findings indicate even less of a disturbance in the normal mechanisms in these control animals than was the case with the phosphatide fractions from the tubercle bacilli.

Because of the intraperitoneal route for the injections and the striking findings with the phosphatide fractions limited essentially to the local manifestations in the peritoneal cavity, the greatest interest attaches to the survey of this region, particularly the peritoneum and omentum. Both parietal and visceral peritoneum in five of the animals were glistening, smooth, and uninvolved throughout; only in Rabbit R 245, of the second group, were there found several strong, fibrous adhesions extending between the site of injection in the left abdominal wall and the serosa of the descending colon; the latter were probably secondary to some trauma incident to one of the injections.

In the two animals receiving lecithin alone (R 243, R 244), the omentum in the gross showed a patchy, dusky, red color, with an increase in milk bodies, but lacked the extensive nodular thickening present in the animals receiving the phosphatide fractions. Supravital studies of films of omentum spread on neutral

red-Janus green slides showed a dilatation of the omental capillary bed accounting for the gross appearance. The reaction of the adventitial cells along the small veins was particularly prominent. The milk spots were focal accumulations of highly phagocytic clasmotocytes, their vacuoles reacting brilliantly with the neutral red. The phagocytized debris was globular but clearly not red cells. No monocytes or epithelioid cells could be identified as such. These identical preparations were subsequently fixed *in situ* in formalin, which faded the neutral red of the clasmotocytes, and were then subjected to staining with Sudan III. On reexamination of the same areas previously studied in the supravital, it was

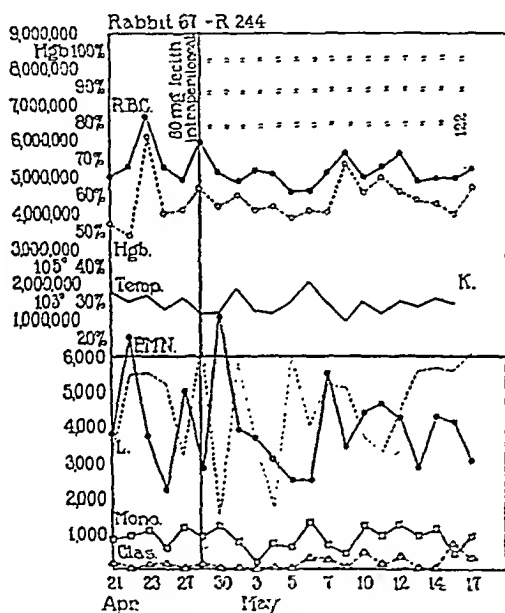


CHART 6.

found that the vacuoles formerly reacting to the neutral red also reacted to the fat stain, quantitatively, indicating a specific response to the foreign phospholipin of the strain of cells of the connective tissues chiefly responsible for the handling of non-specific debris.

The human tubercle bacilli, Strain H 37, used in the last four animals of this control group, were grown under the same conditions as those used for the chemical analyses yielding the proteins and phosphatides of this experiment, and were, indeed, dried weights from identical lots used by Dr. Johnson and Dr. Anderson. Known weights were inactivated at 60° for 1 hour and the individual dosage in all four animals was the same though the total dosage varied as indicated in Table I.

It was the desire to compare the relative reactions of known weights of dead bacilli alone *via* the peritoneal route with comparable dosages in the presence of a non-specific phospholipin.

The two animals receiving lecithin in dosages comparable to those of the phosphatide fractions, plus known quantities of inactivated human tubercle bacilli (R 245, R 246), also showed the evidence of dilated vessels and increased milk bodies in the omentum. However, supravital studies of omental spreads from these rabbits showed the predominating reaction in R 245 to be the typical epithelioid cells, with clasmatoctyes, as in the preceding group, stimulated though

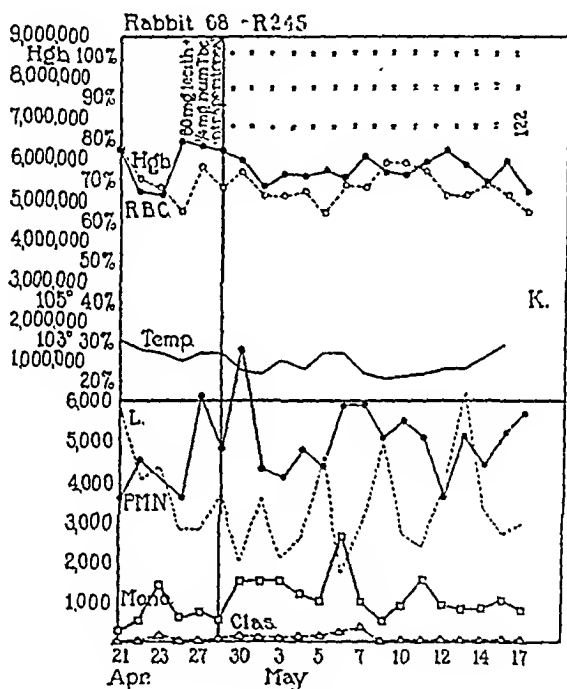


CHART 7.

less in number; while in R 246, the predominant reaction was clasmatoctytic with a less pronounced response of epithelioid and giant cells.

In the two animals receiving dead bacilli only (R 247, R 248), the omentum had the gross appearance already noted. Supravital studies showed the more densely cellular areas to be predominantly clasmatoctytic, though without the excessive phagocytosis of fat globules so prominently a part of the picture seen in the preceding four rabbits. The Sudan III preparations showed many of the cells characteristic of the clasmatoctye group with fine orange globules and an occasional larger fat body, but the majority of the cells did not react. This was in particularly striking contrast quantitatively to the studies in rabbits receiving the

lecithin. Typical epithelioid cells and giant cells of the Langhans type while present were a minority finding in these omenta subjected to dead bacilli.

Thus it will be seen that the intraperitoneal injections *per se* caused more or less of a vascular dilatation of the omentum, giving rise to a characteristic dull pink coloration, with a local cellular stimulation predominantly clasmatocytic in all the animals with the exception of R 245. Those rabbits receiving only lecithin showed no reaction of the monocytic strain of cells which could be identified with the usual criteria of the supravital technic, but rather there was a profound phagocytic response on the part of clasmatocytes as shown by surveys with

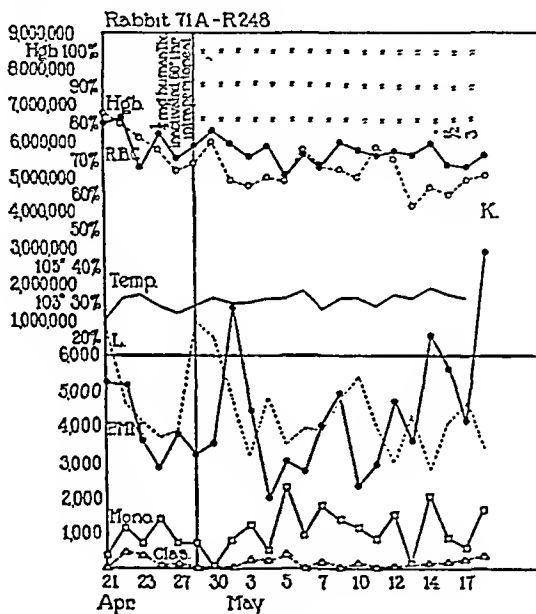


CHART 8.

Sudan III. The response of epithelioid cells was minimal except in the case of Rabbit R 245, in which both lecithin and bacilli had been given, and here there was nothing comparable in the extent and specificity of the reaction to that seen in any of the animals receiving the special phosphatide fractions from the tubercle bacilli.

These reactions of peritoneum and omentum are an adequate index of the general findings in the other tissues of the several groups. The mesentery showed some increase of cellular bodies comparable histologically to those described in the omentum. The mesenteric lymph glands in all animals were swollen, tense, and on section a copious flow of opaque fluid was released, which coagulated quickly

on standing. There was in every instance a great excess, four to five per oil immersion field, of typical clasmatoocytes loaded with debris, but in all supravital studies only two typical epithelioid cells were seen (R 245) and no giant cells. The bone marrows in every animal were normal in their hemopoietic activity, and no epithelioid or giant cells were found in any case. There was only one instance in the control group of six animals in which there was an acute splenic tumor. The spleen of R 244 weighed 2.4 gm., but on supravital study showed only the normal cellular differential except for numerous red blood cells. Only one spleen (R 245) showed epithelioid cells, three in number.

Pleural cavity, lungs and heart, liver, intestinal tract, kidneys, from the animals receiving lecithin were negative in the gross and microscopically.

Both animals receiving lecithin plus bacilli showed some increase of monocytes in the lung with occasional typical epithelioid cells, but with the predominant cell being the clasmatoocyte. The large intestine in R 245 was involved, possibly at the site of an injury incident to the injection, the small intestine being unaffected throughout.

Rabbit R 247, receiving dead bacilli only, showed a few epithelioid cells in the lung preparations, but in R 248 none were found, the usual clasmatoocyte predominance being the obvious finding in both cases.

Certainly it may be accepted that dead bacilli alone in repeated doses and in the amounts cited, when injected intraperitoneally, can excite in the peritoneal cavity only a very limited development of the cellular pathology pathognomonic of tuberculosis; that lecithin is quite incapable of inducing other than a non-specific phagocytic response; and that the combination of lecithin and dead bacilli together reacts essentially independently with only a minimal response of epithelioid cells.

From the literature it is clear that the intravenous injection of dead tubercle bacilli produces in the lungs two distinct reactions: first, the so called interstitial pneumonia, primarily a proliferation of clasmatoocytes; and second, small foci of typical epithelioid and giant cells. The protein and phosphatide fractions from tubercle bacilli here used sharply differentiate and separate these two reactions.

It may not be surprising to find the occasional epithelioid cell in other pathological cellular responses, for in the limited surveys with the supravital technic already made, they have been found in Hodgkin's disease and in the septa of certain tumors. The exact nature and distribution of the specific stimulant, possibly a lipoid, remain to be determined. However, the very special relationship

which this reaction bears to tubercular infection, and the apparent symbiosis of tubercle bacillus and epithelioid cell, present an approach to this disease which is thought at present to be significant.

In the further studies with the chemical products from the tubercle bacilli it will be important to analyze the biological reactions in terms of the phagocytic mononuclear cells of the connective tissues, with the possible differentiation functionally into monocytes, epithelioid cells, and clasmatoctes.

SUMMARY.

1. The clasmatoctes, the cell with the power of fragmenting tubercle bacilli, the cell making the lesion of the so called interstitial pneumonia, has been shown to be the overwhelming response to the special protein fractions, 304 and 903. Multiple hemorrhages, high fever, and toxicity have marked the use of these fractions in every instance.

2. The epithelioid and the giant cell of the Langhans type, making typical tubercular tissue, have been the massive and specific response of the peritoneal cavity to intraperitoneal injections of the phosphatide fractions, A-3 and A-4. These fractions have been entirely non-toxic in the dosages used.

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EXPLANATION OF PLATES.

PLATE 26.

FIG. 1. Typical resting fibroblast from the omentum of a rabbit, R 158, which had received 13 intraperitoneal doses of the phosphatide fraction A-4 on successive days, and was killed 6 days after the last injection. Stained in supravital neutral red. Magnification about 1940, as indicated by the red cell near Fig. 5.

FIG. 2. Stimulated, round clasmatoocyte from the same preparation as the cell of Fig. 1.

FIG. 3. Epithelioid cell, stained supravitaly with neutral red, taken from the peripheral blood of a rabbit, R 19, 16 days after the injection of 1 mg. of living bovine tubercle bacilli into the ear vein. The round, unstained bodies are fat. The chart of the blood of this animal is shown in another paper in this *Journal* (11) as Chart 5; there were 6000 monocytes in a c.mm. of the blood at the time, Dec. 2, when this cell was drawn. Magnification shown by the red cell.

FIG. 4. Slightly stimulated clasmatoocyte from the same preparation as the cell of Fig. 1.

FIG. 5. Typical epithelioid cell, from the same preparation as the cell of Fig. 1, showing the rosette, with the carmine tone in supravital neutral red, characteristic of the type.

FIG. 6. Drawing of lymph cords and sinuses from the center of a mesenteric lymph gland of a rabbit, R 131, after 26 injections of the protein fraction 903. Tissue fixed in Zenker-formol. Stained with hematoxylin and eosin. Drawn with the camera lucida, magnification about 870. The lymphatic endothelium and the clasmotocytes which have become free from it are marked by their content of the debris of red cells from a hemorrhagic area which was being drained. *a*—reduplication of endothelium with the outer cell becoming rounded up; *b*—a denuded area where the endothelium has desquamated; *c*—an endothelial cell stretching across the sinus. In the center is a tiny lymph cord with a perivascular clasmotocyte; to the left are free clasmotocytes in the lymph cord.

PLATE 27.

FIG. 7. Photograph of a section of the lung from Rabbit R 130, which had received 12 intravenous injections of the protein fraction 304. Animal found dead the morning after the last dose. Shows the maximum thickening of the septa with clasmotocytes, the type proved by supravital studies since the cells were still living. All the tissues were fixed in Zenker-formol and stained in hematoxylin and eosin. \times about 90.

FIG. 8. Photograph of the duodenum of a rabbit, R 159, which had received 13 intraperitoneal doses of the phosphatide fraction A-4, and was killed 4 days after the last dose. To show the extent of the serosal lesion. The mass of the tissue is of epithelioid cells, with the dark spots in the center of lymphocytes and two large masses of necrosis to the left. \times about 9.

PLATE 28.

FIG. 9. Photograph of the retroperitoneal connective tissue, in the edge of the psoas muscle near one kidney, of a rabbit, R 129, which had 13 doses of the protein fraction 304. Killed 24 hours after the last dose. The tissue is hemorrhagic and edematous, and shows a stimulation of the connective tissue cells which were predominately clasmotocytic in the supravital studies. \times about 455.

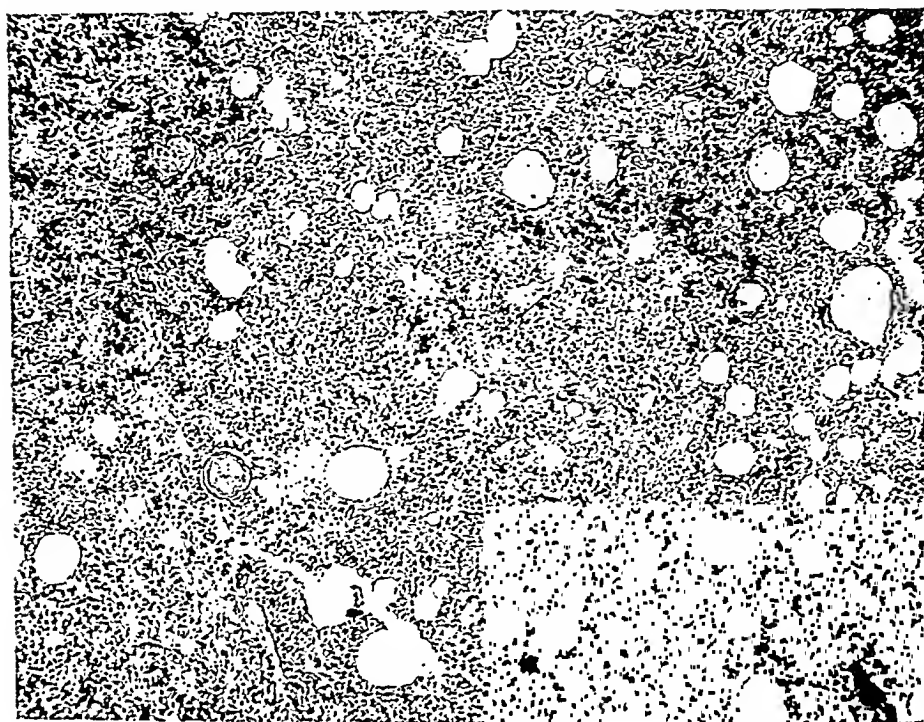
FIG. 10. Photograph of the connective tissue near the bladder from a rabbit, R 153, which received 14 intraperitoneal doses of the phosphatide fraction A-3, and was killed 24 hours after the last dose. It shows a reaction predominately of epithelioid cells and giant cells of the Langhans type. \times about 455.

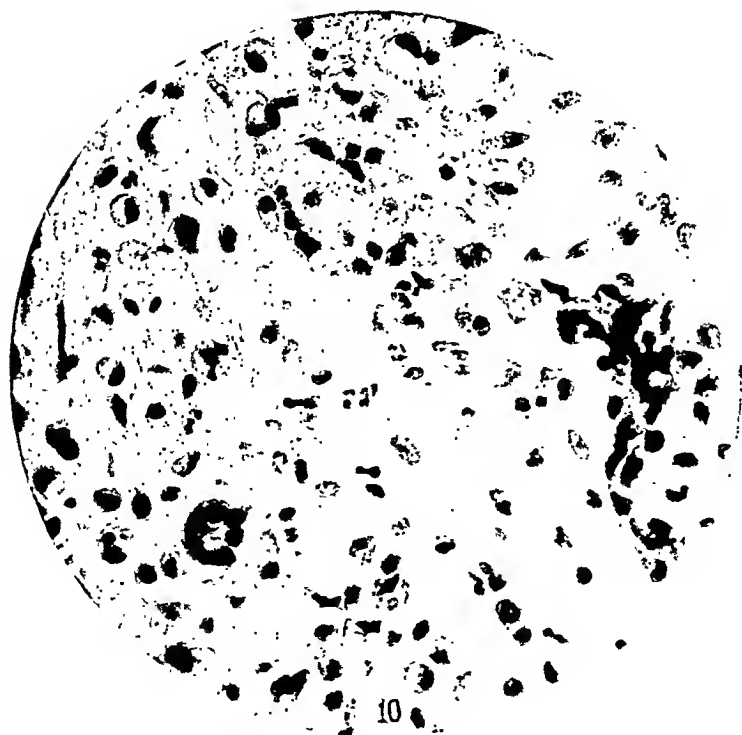
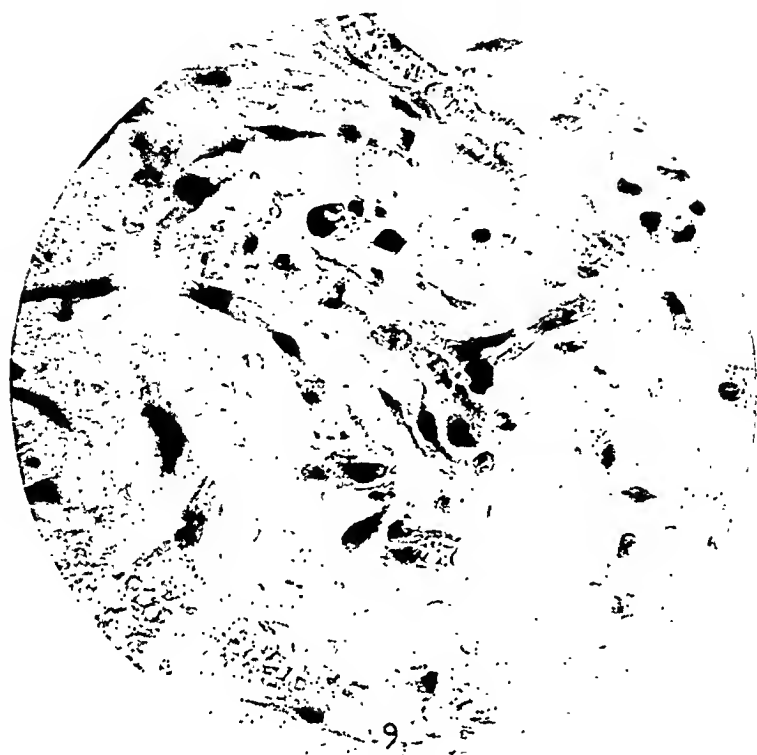
PLATE 29.

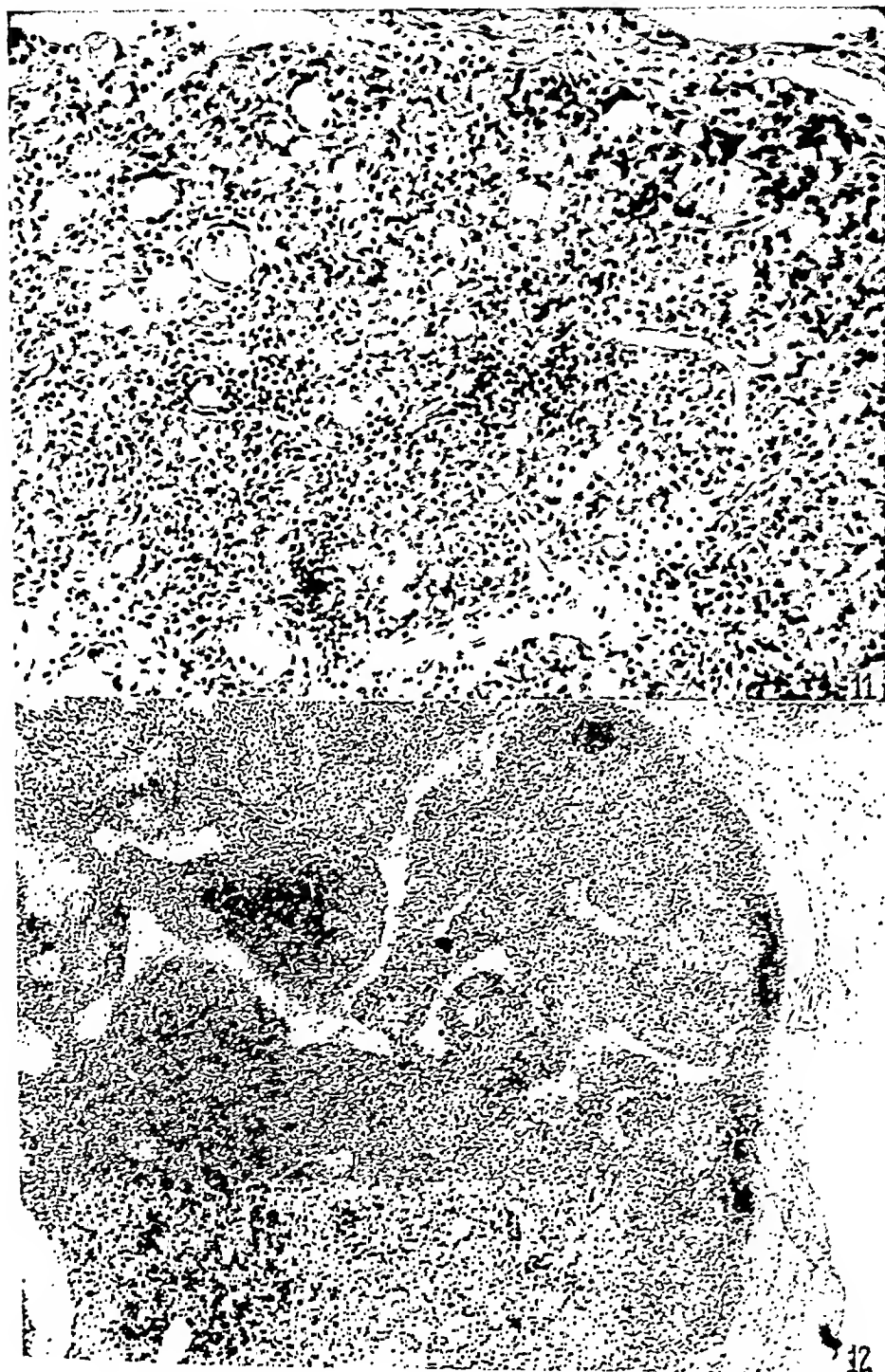
FIG. 11. Photograph of the omentum of a rabbit, R 153, which had received 14 intraperitoneal doses of the phosphatide fraction A-3, and was killed 24 hours after the last dose. It shows a reaction predominately of the giant cells of the Langhans type. There are many blood vessels and some clumps of lymphocytes. \times 195.

FIG. 12. Photograph of the mesenteric lymph gland of a rabbit, R 158, which had received 13 intraperitoneal doses of the phosphatide fraction A-4, and was killed 6 days after the last dose. The peripheral follicles with pale centers show extensive involvement with typical epithelioid cells. \times about 90.









THE EFFECT OF SERUM UPON THE GERMICIDAL ACTION OF SOAPS.

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That serum inhibits the germicidal action of soaps was shown by Noguchi (1907) and by Landsteiner and Ehrlich (1908). Further observations were later made by Lamar (1911) and by Walker (1924), Walker being the first to show that the effect of serum upon soaps varied both with the soap used and with the organism.

In all these investigations, the effect of the pH was neglected. Serum that has come to equilibrium with the CO₂ of the air has a pH of 8.6 to 8.8. As the soaps having twelve or more carbon atoms are strongly hydrolyzed, their solutions are more or less alkaline. Hence, with no adjustment of the pH, the test fluid will have a reaction of pH 8.6 to 9.5 or more.¹ As a previous investigation (Eggerth, 1926) had shown that the germicidal action of soaps in salt solutions was greatly modified by the pH, it was determined to study the action of serum on soaps over a wide pH range.

EXPERIMENTAL.

Soaps of the following normal fatty acids were used: caprylic, capric, lauric, myristic, palmitic, and oleic. The soaps were prepared by adding the theoretical quantity of fatty acid (Eastman's) to $N/5$ KOH. Kahlbaum's sodium oleate was used. Just before each experiment, serial dilutions of the soap used were prepared in sterile $N/10$ NaCl solution.

The test organisms were: *Streptococcus pyogenes* ("Gay" strain); *B. diphtheriae* ("Park-Williams No. 8"); *B. typhosus* ("Pfeiffer" strain); and *Staphylococcus aureus* (old laboratory culture). These are the same organisms that were used in the previous investigation (Eggerth, 1926).

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¹ The effect of the addition of boric acid to serum-soap mixtures, first described by von Liebermann and von Fényvessy (1909), is due to the shift in the pH.

Fresh, sterile sheep serum was used. To every 10 cc. of serum, 1.0 cc. of $N/5$ HCl was added, to neutralize the $NaHCO_3$ present. The serum then stood overnight to allow the escape of the liberated CO_2 ; the pH was then about 7.2 to 7.6. It was then divided into five or more portions, and each portion adjusted with acid or alkali to the desired pH, the colorimetric method of Cullen (1922) being used to determine the end-point. The volumes of the different portions were then equalized with sterile $N/10$ NaCl. When it was desired to dilute the serum, this was done with sterile phosphate-glycine buffer mixtures of the corresponding pH. These buffers have a salt content of $N/10$; their composition is given in the first article of this series (Eggerth, 1926).

The serum mixtures were then inoculated with the test organism. The inocula

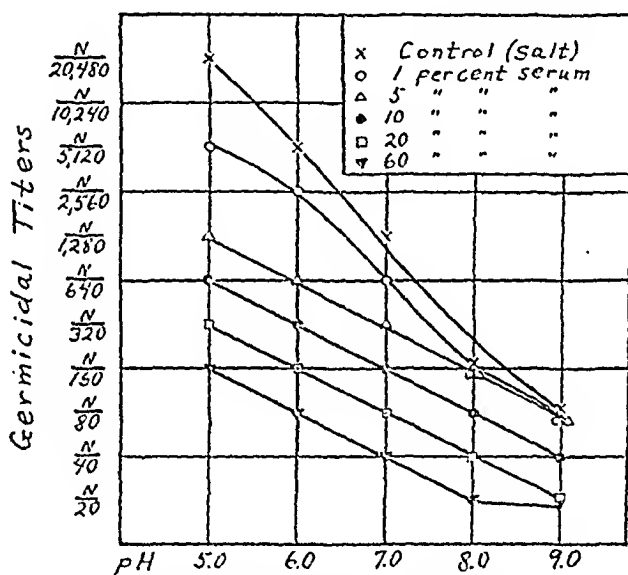


FIG. 1. The germicidal titers of laurate for *Staphylococcus*. The incubation period was 2 hours at $37^{\circ}C$.

were such that each 0.5 cc. of test fluid contained 0.04 cc. of broth culture of *Streptococcus pyogenes* or *B. diphtheriae*, or 0.02 cc. of *Staphylococcus aureus* or *B. typhosus*. Then 0.5 cc. quantities were pipetted into series of small test-tubes. To each tube, 0.5 cc. quantities of soap dilutions were then added. (When a final serum concentration of 60 per cent was desired, 0.75 cc. of adjusted serum and 0.25 cc. of soap dilution were used.) Finally each tube was rotated while in a slanting position and placed in the water bath at $37^{\circ}C$. At the end of 30 minutes, 2 hours, and 18 hours, a 4 mm. loopful from each tube was subcultured on plates of blood agar (*Streptococcus* and *B. diphtheriae*) or plain agar (*Staphylococcus* and *B. typhosus*). At the close of the experiment, the pH of each tube was tested with the appropriate indicator. When substances other than serum (egg white, gela-

tin, etc.) were tested, the same general procedure was followed. The final concentration of salt in the tubes was $N/10$ or a little greater.

The results of some typical experiments are shown in Figs. 1 to 3. In each case, the curve for a particular concentration of serum bears a definite relationship to the curve obtained for salt solution (buffer) alone. Thus, wherever the titer in salt solution alone is high, even small percentages of serum greatly diminish that titer. Wherever the titer in salt solution is low (less than $N/320$), small percentages of added serum leave the titer practically unchanged.

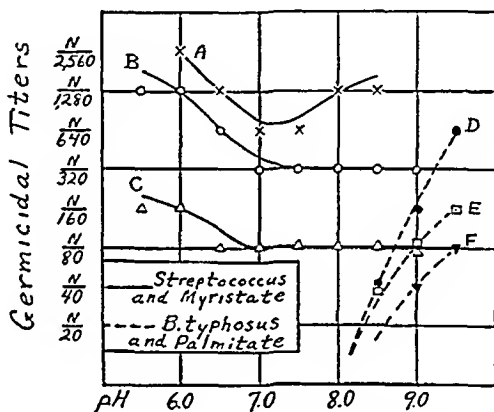


FIG. 2. The germicidal titers of myristate for *Streptococcus*: A, salt control; B, 10 per cent serum; C, 60 per cent serum. The germicidal titers of palmitate for *B. typhosus*: D, salt control; E, 10 per cent serum; F, 60 per cent serum. The incubation period was 2 hours at 37°C .

Thus small additions of serum flatten the curves and make them more nearly horizontal. If further larger amounts of serum are added, the titers are reduced all along the line.

In the combination of *Staphylococcus* with laurate, shown in Fig. 1, the salt solution control makes a steep curve; the ratio of the titer at pH 5.0 to the titer at pH 9.0 is 256:1. The addition of 5 per cent of serum to the soap diminishes the titer at pH 5.0 from $N/20,480$ to $N/1,280$, while the titer at pH 8.0 to 9.0 is unchanged, and the ratio is now only 16:1. Further additions of serum now diminish the titer equally at all pH; the curves for 5, 10, 20, and 60 per cent

of serum are parallel. Other combinations that, in the presence of serum, gave curves similar to those of Fig. 1 were: *Staphylococcus* with caprylate, caprate, and myristate; *B. typhosus* with caprylate and caprate; *B. diphtheriae* with caprylate, caprate, and laurate; *Streptococcus* with caprylate, caprate, and laurate.

In the combination of *Streptococcus* with myristate, shown in Fig. 2, the control curve for salt solution is comparatively horizontal.

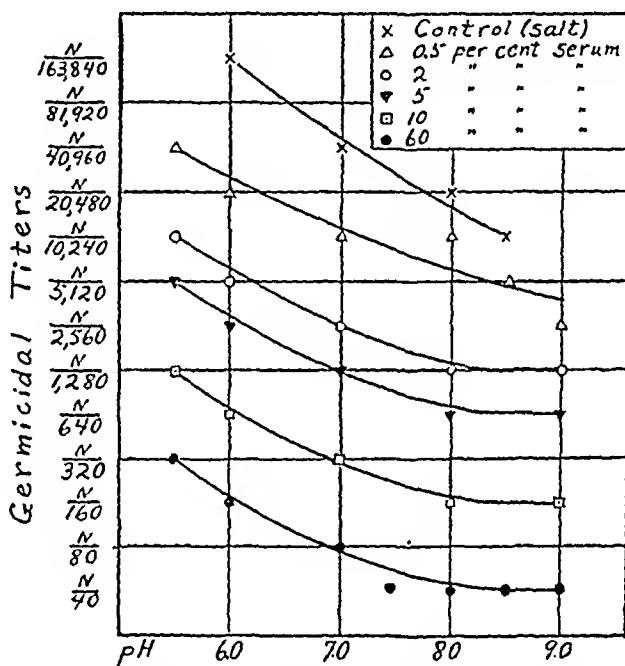


FIG. 3. The germicidal titers of oleate for *Streptococcus*. The incubation period was 2 hours at 37°C.

The addition of serum reduces the titer approximately equally at every pH, so that the serum-soap curves have about the same shape and inclination as the control. The following combinations behaved similarly to this one in the presence of serum: *Streptococcus* with palmitate, and *B. diphtheriae* with myristate and palmitate.

In Fig. 2 are also shown the curves for *B. typhosus* and palmitate. Here the curve of the control rises steeply toward the alkaline side; the addition of serum inclines the curve toward the horizontal. Similar results were obtained with *B. typhosus* and laurate, myristate, and oleate.

Fig. 3 gives the effect of serum upon *Streptococcus* and oleate. Here the titer in salt solution is high at every pH, though highest in the acid range. Even 0.5 per cent of serum diminishes the titer from N/163,840 to N/10,240 at pH 6.0, and from N/10,240 to N/5,120 at pH 8.0 and 8.5. Further additions of serum diminish the titer equally at every pH.

It is an interesting fact that serum increases both the acid and the alkali tolerance of bacteria. Thus, in salt solution (phosphate-glycine buffer) this strain of *Streptococcus* invariably dies within 30 minutes at pH 5.5 and pH 9.0 at 37°C., while in 0.5 per cent serum it survives for 18 hours at the same reactions. (This organism has become more sensitive to acid and alkali during the past year, hence the tolerance is now different from that reported previously.)

DISCUSSION.

Several investigators have attempted to explain the mechanism of the inhibiting effect of serum upon soap. Von Liebermann (1907) ascribed the inhibitory action to the calcium of the serum, and von Liebermann and von Fenyvessy (1909) suggested both calcium and cholesterol as active constituents of the serum. Sachs (1908), on the other hand, believed the serum proteins to be responsible. Von Korányi (1908) showed that the addition of serum to soap solution caused a slow increase of the surface tension of the soap solution, and suggested this as the cause of the inhibitory effect of the serum. Walker (1924) again suggested calcium as the effective substance, although Lamar (1911) had denied this, and Clark, Zinck, and Evans (1921) stated that calcium played only a minor part in the inhibitory action of serum.

Inasmuch as the mechanism of soap inhibition by serum is of considerable interest, this problem was taken up. Preliminary experiments showed that the active principle was heat-stable; when diluted and boiled, serum is still inhibitory. The active principle does not diffuse through collodion membranes whose permeability is such that they hold protein back. When serum is fractionated by dialysis or by ammonium sulfate precipitation, each fraction is active, and the activity varies approximately with the protein content of that fraction.

The calcium of the serum undoubtedly plays some part, but a minor one, as was also found by Clark, Zinck, and Evans (1921) in their studies on soap hemolysis. When oxalated serum was compared with normal serum, the germicidal titers with the two sera were exactly the same at every pH in 6 experiments, as follows: *Streptococcus* with laurate and myristate; *Staphylococcus* with laurate and myristate; *B. typhosus* with laurate and oleate. In 3 other experiments, the oxalated serum gave a soap titer twice as high as the normal serum at every pH, as follows: *Streptococcus* with oleate (2 experiments) and *Streptococcus* with laurate. A 10 per cent concentration of serum was used for these experiments.

TABLE I.

Germicidal Titers of Potassium Laurate for Staphylococcus in the Presence of Calcium Chloride.

pH	Acetate-asparagine buffer			Phosphate-glycine buffer		
	Control	1-5,000 CaCl ₂	1-1,250 CaCl ₂	Control	1-10,000 CaCl ₂	1-2,000 CaCl ₂
5.0	N/20,480	N/20,480	N/20,480	N/20,480	N/20,480	N/20,480
6.0	N/5,120	N/5,120	N/320	N/5,120	N/5,120	N/160
7.0	N/1,280	N/320	N/80	N/1,280	N/320	N/40
8.0	N/320	N/160	N/20	N/320	N/160	N/40
9.0	N/160	N/80	N/20	N/160	N/80	N/40

The period of incubation was 2 hours; the temperature, 37°C.

In another series of experiments, varying quantities of CaCl₂ were added to buffer solutions without serum, and the soap titers determined. As the phosphate-glycine buffer precipitates the calcium in neutral and alkaline reactions, parallel experiments were undertaken with a special buffer mixture containing N/10 potassium acetate and N/10 asparagine. This buffer was adjusted to the desired pH by means of acetic acid or potassium hydroxide. As is shown in Table I, concentrations of 1-5,000 to 1-10,000 of CaCl₂ definitely lower the titer, but only in the neutral and alkaline ranges. In this respect the effect is quite different from that of serum. Larger amounts of calcium produce an even more decided lowering of the titer, except at pH 5.0.

Table I shows that it does not make any difference whether the buffer precipitates the calcium or not. In the case of the phosphate-glycine buffer, either a double decomposition occurs, and calcium laurate is formed, or the precipitated calcium phosphate adsorbs the laurate; in either case the soap is removed from solution.

The amount of calcium in human blood serum is given as 10 to 12 mg. per 100 cc. If it is assumed that sheep serum contains the same amount, then a 60 per cent serum would contain as much calcium as a 1-6,000 solution of CaCl_2 ; while 10 per cent serum should contain as much as a 1-36,000 solution. From Table I it is apparent that such small amounts of calcium can play only a very minor part in the serum inhibition of soaps, and then only in the neutral and alkaline reactions.

The serum protein is undoubtedly more important. Unfortunately this cannot be conclusively proved directly, as no method is known for completely freeing serum protein from lipoid without at the same time denaturing the protein and making it water-insoluble. Extraction with ether, as is well known (Maclean, 1918), removes only a small part of the lipoid. A method which removes a large part of the serum lipoid without denaturing the protein is that of Hardy and Gardiner (1910). This method, modified somewhat to serve the present purpose, is as follows:

To 1 volume of serum at 0°C ., 12 volumes of absolute alcohol at 0°C . are added. The mixture is held at 0°C . for 1 hour, with occasional shaking; it is then filtered with suction. The precipitate is washed, first with a mixture of cold alcohol and ether, then with pure ether. In all these operations, the temperature is kept at or near 0°C . A dry powder is obtained which is readily and completely soluble in $\text{N}/10$ NaCl solution.

In Figs. 4 and 5 are shown the titers given by such a "defatted" serum having a concentration of 0.7 per cent dry protein (representing 10 per cent of original serum). The acid titers are somewhat higher in both cases than in 10 per cent normal serum, while the alkaline titers are only a little higher. It should be remembered that this method does not remove all the serum lipoid; repeated extraction with hot alcohol is necessary for complete defatting. Hence it is impossible to tell whether the activity of this partially defatted serum

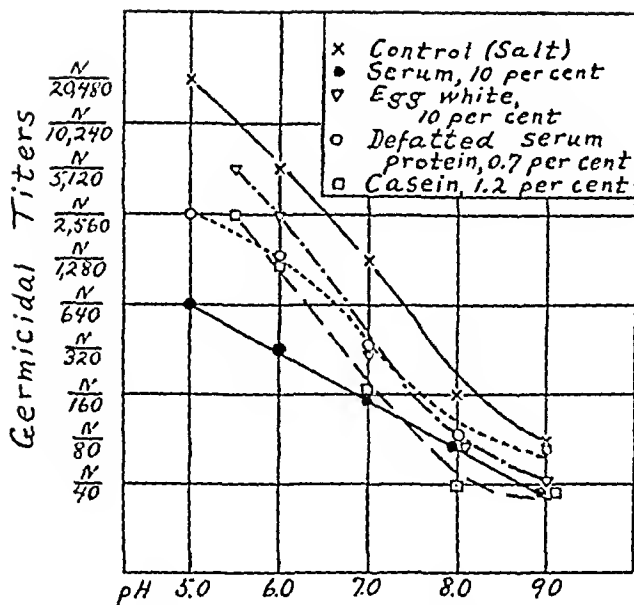


FIG. 4. The germicidal titers of laurate for *Staphylococcus* in the presence of various proteins. The incubation period was 2 hours at 37°C. Crystalline egg albumin in a concentration of 1.5 per cent; gelatin, 2 per cent; and peptone, 2 per cent, each gave a curve exactly like that of the salt control.

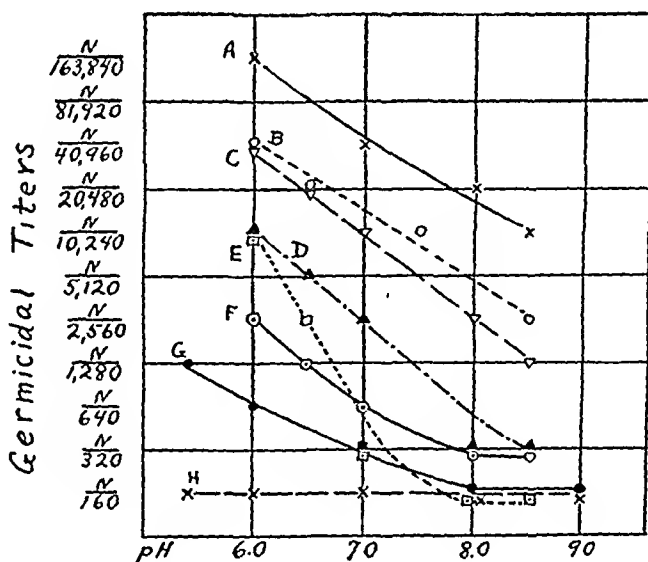


FIG. 5. The germicidal titers of oleate for *Streptococcus* in the presence of various proteins. A, salt control; B, crystalline egg albumin, 1.5 per cent; also peptone, 2 per cent; C, gelatin, 2 per cent; D, egg white, 10 per cent; E, casein, 1.2 per cent; F, defatted serum protein, 0.7 per cent; G, serum, 10 per cent; H, egg yolk, 1 per cent. The period of incubation was 2 hours at 37°C.

represents the true activity of the protein or that of the residual lipoids.

A number of other protein substances were tested (Figs. 4 and 5). Unaltered egg white, in a concentration of 10 per cent, gave quite different curves from 10 per cent serum, the titers in acid reactions being very much higher than with serum. Crystalline egg white was prepared by the method of Hopkins and Pinkus (1898), recrystallized three times, and dialyzed. In a concentration of 1.5 per cent, it showed no inhibitory action whatever for laurate with *Staphylococcus*, and only slight inhibition of oleate with *Streptococcus* (Fig. 5). Clark, Zinck, and Evans (1921), using crystalline egg albumin in hemolytic experiments with sodium oleate, found that this substance, instead of inhibiting, augmented hemolysis by the soap; this was probably a pH effect, as the crystalline albumin is acid.

Gelatin (freed from calcium by the method of Loeb (1919)), in 2 per cent concentration, and peptone (Parke, Davis and Co.), also in 2 per cent concentration, behaved like the crystallized egg albumin; no effect of these substances was noticed on the action of laurate with *Staphylococcus*, while they only slightly inhibited oleate with *Streptococcus* (Fig. 5).

Casein was prepared from fresh milk by the method of Van Slyke and Baker (1918), and was carefully freed from lipid by repeated extraction with warm alcohol and ether. In a concentration of 0.75 to 2 per cent, it proved to be an actively inhibitory substance, but the curves are decidedly different from those of serum (Figs. 4 and 5). The alcohol-soluble protein associated with casein (see Osborne and Wakeman, 1918), when suspended in the buffer solutions, proved to be very strongly inhibitory to the germicidal action of soaps.

Egg yolk also was found to be a powerfully inhibitory substance (Fig. 5). With only 1 per cent egg yolk, the titer of laurate for *Staphylococcus* was reduced to $N/80$ at every pH between 5.0 and 9.0 (not shown in the figure). The activity of egg yolk is probably mostly due to its high lipid content.

Washed red cells, added to the soap solutions, gave curves almost identical with those of serum.

From these experiments it will be seen that every protein substance tested was more or less inhibitory to the oleate-*Streptococcus* combina-

tion; crystalline egg albumin, gelatin, and peptone, however, failed to inhibit the action of laurate upon *Staphylococcus*. It is undoubtedly a significant fact that all the curves obtained for protein substances run more nearly parallel to the curve for salt solution than they do to the curves for serum.

The lipoids of the serum have hitherto not been definitely related to the soap-inhibiting property. Von Liebermann and Fenyvessy (1909), it is true, reported that cholesterol diminished the hemolytic power of sodium oleate, but Clark and Evans (1921), with more accurate titrations, failed to confirm this. Sachs (1908) extracted lipoids from serum by means of ether, and found that the lipoids alone had no effect on soap hemolysis. Clark and Evans (1921) found the petroleum ether extractives of serum to be inactive, as likewise commercial lecithin.

The experiments shown in Figs. 6 to 8, however, indicate that the lipoids are actively inhibitory to the action of soaps. In the experiments of Figs. 6 and 7, the lipoids were tested in the form of aqueous emulsions, which were prepared as follows:

1. Cholesterol, cholesteryl oleate, and olive oil were made up in a 0.5 per cent solution in acetone and added to the soap dilutions. The acetone was then driven off by heat, and the lipid-soap emulsions added to the inoculated buffers. The cholesteryl oleate was prepared by the method of Hürthle (1895).

2. Lecithin was prepared from egg yolks, as commercial lecithin was found to be very impure. The yolks were dried, by two extractions with acetone; then they were extracted twice with alcohol at 40°C. The alcoholic extracts were united and evaporated at 45°C.; the residue was dissolved in ether, filtered, and precipitated with acetone. This precipitate was then twice dissolved in ether and precipitated with acetone. The final product was very pale yellow in color and dissolved completely in alcohol. It probably contained considerable cephalin; but as cephalin is also found in serum, there seemed to be no reason for removing it. This lecithin was readily emulsified by stirring in water.

3. Oleic acid emulsion was prepared by adding HCl to sodium oleate solution. Oleic acid and sodium oleate are practically non-toxic for *Staphylococcus* (Walker, 1924; Eggerth, 1926).

4. The serum lipoids were prepared by extracting serum several times with 5 volumes of warm alcohol and then ether: the filtrates were evaporated and the residue dissolved in alcohol and filtered. The alcoholic solution was evaporated, with additions of water from time to time. When free from alcohol, the volume was made up to that of the original serum.

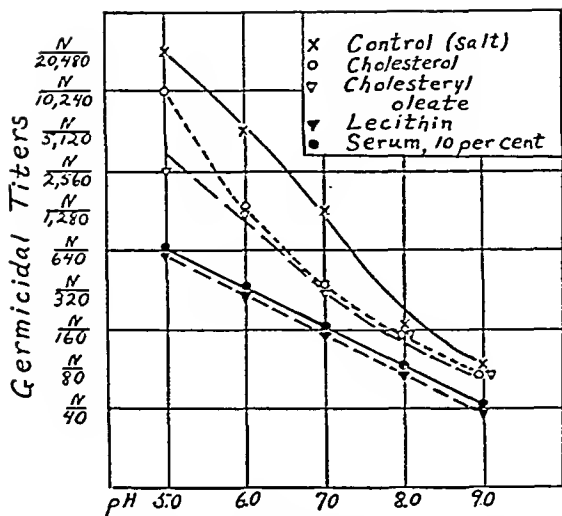


FIG. 6. The germicidal titers of laurate for *Staphylococcus* in the presence of lipid emulsions. The lipid emulsions shown are all in a concentration of 1-1,000. An oleic acid emulsion of the same concentration gave a curve identical with that shown for cholesteryl oleate, as did also olive oil. Serum lipids, in a concentration equivalent to 10 per cent serum, also gave a curve identical with that for cholesteryl oleate. The incubation period was 2 hours at 37°C.

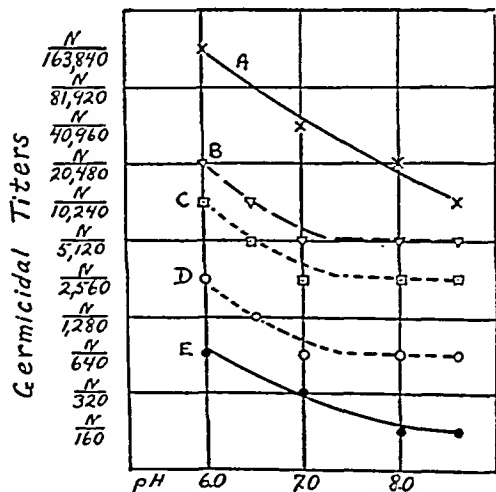


FIG. 7. The germicidal titers of oleate for *Streptococcus* in the presence of lipid emulsions. A, salt control; B, cholesteryl oleate, 1-1,000; also olive oil, 1-1,000; C, lecithin, 1-5,000; D, lecithin, 1-1,000; E, serum, 10 per cent. The incubation period was 2 hours at 37°C.

Figs. 6 and 7 show that the lipoids in aqueous emulsions all inhibit the action of soaps. This is especially true of lecithin. A 1-1,000 lecithin emulsion gave a curve identical with that of 10 per cent serum (Fig. 6). Even very dilute emulsions of lecithin were appreciably active; thus, a 1-8,000 emulsion gave a curve identical with that of 1-1,000 cholesteryl oleate (Fig. 6). This outstanding activity of lecithin is undoubtedly due to the basic nature of its molecule, which favors union with the fatty acids.

As the lipoids in serum are present as a highly dispersed colloidal solution, it seemed desirable to test them in aqueous solution. Two such preparations could be obtained. (1) Oleic acid is highly soluble in casein solutions. A 5 per cent casein solution containing $N/40$ sodium oleate remains perfectly clear when brought to pH 5.5, whereas the solubility of oleic acid in buffer solutions at pH 5.5 is $N/5,120$. In the experiments shown in Fig. 8, the test fluids contained 0.75 per cent of casein and $N/320$ of oleate; they remained water-clear at all reactions indicated. (2) Lecithin is highly soluble in bile salts, as shown by Long and Gephart (1908). A sample of sodium taurocholate (Eimer and Amend, "pure") was found to contain an ether-soluble impurity which interfered with the solution of lecithin and was also toxic for *Streptococcus*. The commercial preparation was purified by dissolving it in alcohol containing 2 per cent of sulfuric acid, then precipitating with ether. The precipitate was twice dissolved in alcohol and precipitated with ether, then dried over sulfuric acid. A concentrated solution of taurocholic acid or its salt will dissolve a large quantity of lecithin. Long and Gephart (1908) state that 5 gm. of "bile salt" will dissolve 4.2 gm. of lecithin; with the preparations used in these experiments, even larger amounts of lecithin were readily dissolved. The solutions are perfectly clear and stable at every pH tested and at all dilutions.

The results of these experiments are shown in Fig. 8. The solution of oleic acid-sodium oleate in casein is more inhibitory to the germicidal action of laurate upon *Staphylococcus* than casein alone or the oleic acid-sodium oleate alone in emulsion form (Fig. 6). The taurocholate alone is quite indifferent to the *Staphylococcus*-laurate combination in the concentration tested (1-500); but when lecithin, in a concentration of 1-750, is dissolved in the taurocholate, the

inhibition is pronounced. The lecithin in solution is more active than the lecithin in emulsion (Fig. 6).

The curve for *Streptococcus* and oleate with taurocholate is rather peculiar. This is because the taurocholate, in a concentration of 1-500, is itself germicidal to this *Streptococcus* at pH 6.0. At pH 6.5 it is not germicidal, but its toxicity is added to that of the soap,

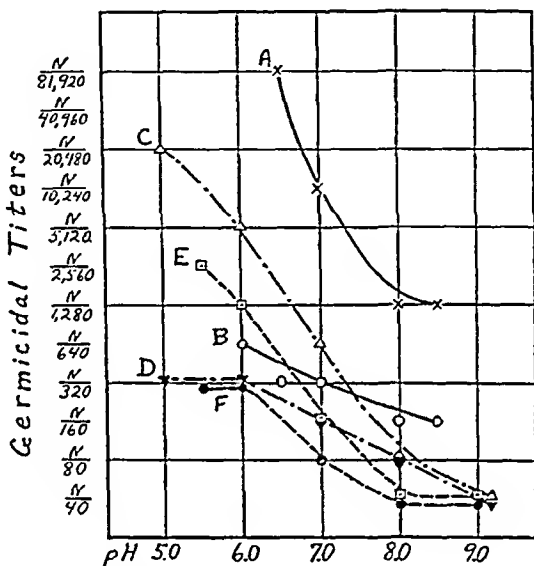


FIG. 8. The germicidal titers of soaps in the presence of lipoids in solution. A, titers of oleate for *Streptococcus* in the presence of 1-500 taurocholate (control); B, titers of oleate for *Streptococcus* in the presence of lecithin, 1-750, dissolved in 1-500 taurocholate. C, titers of laurate for *Staphylococcus* in the presence of 1-500 taurocholate (control); D, titers of laurate for *Staphylococcus* in the presence of lecithin, 1-750, dissolved in 1-500 taurocholate. E, titers of laurate for *Staphylococcus* in the presence of 0.75 per cent casein (control); F, titers of laurate for *Staphylococcus* in the presence of 1-1,000 oleic acid dissolved in 0.75 per cent casein. The incubation period was 2 hours at 37°C.

giving a very high soap titer. At pH 8.0 and 8.5, the taurocholate definitely inhibits the germicidal action of the oleate. But when lecithin was dissolved in the taurocholate solution, a curve was obtained that was identical with that of 10 per cent serum. The lecithin inhibited not only the germicidal action of the oleate, but that of the taurocholate as well.

It was noticed, early in the course of the experiments, that those substances that lower the germicidal titer of the soap, usually increase the solubility of that soap; this increase in solubility was often most marked in the acid reactions. It was often possible to predict the effect of a test substance upon the germicidal titer from the solubility of the soap in that substance. These relationships are shown in Table II.

In determining the solubilities of soaps in serum (Table II) it was found necessary to oxalate the serum to determine solubility at pH 7.0 and more alkaline reactions, otherwise a part of the soap is precipitated by the serum calcium and the results obscured.

A number of interesting facts are brought out by Table II. In the presence of serum at different dilutions and different pH, the ratio of the solubility of the soap to its germicidal titer is remarkably constant, indicating that these phenomena are in some way connected. The corresponding ratios for other test substances vary a great deal; yet it will be observed that every substance that increased the solubility of the soap (or fatty acid) diminished its germicidal titer more or less. The high solubility of oleic acid in casein solution, and of both oleic and lauric acids in lecithin-taurocholate solutions, is noteworthy.

The experiments described above indicate that the serum lipoids and serum proteins both participate in the action of serum upon soap. In unaltered serum it is probable that they largely exist as protein-lipoid compounds. The question still remains, what is the mechanics of their action upon soaps?

Du Noüy (1922, 1926), in his surface tension studies, interpreted the action of serum upon soaps as being due to an adsorption of soap molecules on the surfaces of the huge serum molecules. In this way the soap is bound so that it can no longer concentrate on other surfaces, and the surface tension of the mixture becomes, in a short time, the same as the surface tension of the serum alone. Even very small quantities of serum, 5 per cent or less, suffice to bring this about.

This explanation might be taken as it stands to account for the effect of serum upon the germicidal action of soaps, were it not for two facts: (1) Under certain conditions, serum inhibits the germicidal action very little or not at all (*e.g.*, the effect of 5 per cent serum on

TABLE II.
The Relation of Solubility to Germicidal Titer.

Test substance	Laurate with <i>Staphylococcus</i>				Oleate with <i>Streptococcus</i>			
	pH	Solubility	Germicidal titer	Ratio	pH	Solubility	Germicidal titer	Ratio
Phosphate-glycine buffer.....	5.5	N/5, 120	N/10, 240	1-2	6.0	N/5, 120	N/163, 840	1-32
“ “	7.0	N/1, 280	N/1, 280	1-1	7.0	N/5, 120	N/40, 960	1-8
“ “	9.0	N/320	N/80	4-1	8.5	N/640	N/10, 240	1-16
Serum, 10 per cent.....	5.5	N/2, 560	N/640	4-1	6.0	N/2, 560	N/640	4-1
“ “ “	5.5	N/640	N/160	4-1	6.0	N/640	N/160	4-1
Oxalated serum, 10 per cent.....	7.0	N/640	N/160	4-1	7.0	N/1, 280	N/640	2-1
“ “ “ “	7.0	N/320	N/40	8-1	7.0	N/640	N/160	4-1
“ “ “ “	9.0	N/160	N/40	4-1	9.0	N/640	N/320	2-1
“ “ “ “	9.0	N/80	N/20	4-1				
Egg white, 10 per cent.....	5.5	N/5, 120	N/5, 120	1-1	6.0	N/5, 120	N/10, 240	1-2
Crystalline egg albumin, 1.5 per cent.....	5.5	N/5, 120	N/10, 240	1-2	6.0	N/5, 120	N/40, 960	1-8
Gelatin, 2 per cent.....	5.5	N/5, 120	N/10, 240	1-2	6.0	N/5, 120	N/40, 960	1-8
Taurocholate, 1-500.....	5.5	N/5, 120	N/10, 240	1-2				
Lecithin, 1-750 (in taurocholate).....	5.5	N/160	N/320	1-2	6.0	N/320	N/640	1-2
Casein, 1.5 per cent.....	5.5	N/640	N/2, 560	1-4	6.0	N/40	N/2, 560	1-64

The solubilities and germicidal titers were determined after 2 hours at 37°C. All test substances were made up in the standard phosphate-glycine buffer. The oxalated serum was prepared by adding 0.5 cc. of 2 per cent ammonium oxalate to 10 cc. of serum and centrifuging until clear.

laurate with *Staphylococcus* at pH 8.0 and 9.0, Fig. 1); and (2) certain substances found by du Noüy to be as active as serum in raising the surface tension of soap solutions, such as gelatin and crystalline egg albumin, have little or no effect upon the germicidal action of soap (Figs. 4 and 5).

In the case where lipid emulsions are added to soaps (Figs. 6 and 7), it is obvious that the soap will be distributed between three phases: the suspended lipid, the water, and the bacteria. The concentration of soap in each of these phases will vary, in this particular case, with the solubility of the soap in the respective phases. As the solubility of fatty acids in lipoids is high, then, in acid reactions, the greater proportion of the soap (fatty acid) will be in the suspended lipid: the concentration of germicide in the bacterial protoplasm will consequently be low, and germicidal action will be greatly diminished. On the other hand, salts of the fatty acids (soaps) are not very soluble in lipoids; hence in alkaline reactions the lipid phase will contain only a small part of the soap, and the concentration of germicide in the bacteria will be as great or nearly as great as in the salt control; hence germicidal action will not be appreciably inhibited. Figs. 6 and 7 show that this actually occurs. When lipoids in aqueous solution are the test substances (Fig. 8) the results are the same, and it seems likely that the mechanism also is the same.

When serum and bacteria are added to soap solutions the same thing probably occurs. The soap divides itself between three phases: bacterial protoplasm, water, and serum molecules: the final concentration in each phase will vary with the combination attraction of that phase for the soap. Whether the combination with the serum molecules is one of solution of the soap in colloidal micellæ (as with lipid emulsions) or of chemical combination, or of adsorption in the sense of du Noüy, is immaterial. Crystalline egg albumin and gelatin are less effective than serum because their attraction for soap molecules is less; this is also shown by the fact that neither of them increases the solubility of soap in water.

SUMMARY AND CONCLUSIONS.

1. Far more information about the effect of serum or other substances upon the germicidal action of soaps can be obtained by deter-

mining the germicidal titers over a wide range of pH than by determining the titer at a single pH. In this way a characteristic curve for each test substance is obtained.

2. The curve for a particular concentration of serum bears a definite relationship to the curve for salt solution (buffer) alone. Wherever the titer in salt solution is high, very small amounts of serum greatly diminish that titer. Wherever the titer in salt solution is low, small amounts of serum leave the titer unchanged. Thus small additions of serum flatten the curves and make them more nearly horizontal. If further large amounts of serum are added, a further reduction in titer takes place at all reactions.

3. The calcium of serum has only a very slight effect upon the soap titer.

4. The protein of serum is probably inhibitory to soaps; but the curve for partially defatted serum, and the curves for other protein substances tested, do not run parallel to the serum-soap curves.

5. The various lipoids that are known to be present in serum are inhibitory to the action of soaps, both as emulsions and as clear solutions.

6. The action of serum upon soaps may be regarded as a complex reaction, in which lipoids, protein, and, to a lesser extent, calcium salts take part. Their effect is due to the fact that these substances, by combining with the soaps, remove them from the field of germicidal action.

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THE INFLUENCE OF PROTEIN-FREE LIVER AND SPLEEN EXTRACTS ON THE BLOOD REGENERATION AND RESPIRATORY EXCHANGE OF ANEMIC RABBITS.

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PLATE 30.

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I. INTRODUCTION.

This series of experiments is a continuation of the experimental work upon blood regeneration in rabbits. The purpose of the series was to gain further evidence of the nature of the action of the liver and spleen extracts upon blood regeneration in highly anemic animals as measured by means of heat production.

The respiratory exchange of anemic animals has been investigated by several authors as is shown in the reviews given by Meyer and Du Bois (1), and Tompkins, Brittingham and Drinker (2). With the exception of Bauer (3), most of the authors (4-6, 13) found the respiratory exchange within normal limits. Kraus, Chvostek and Bohland have reported metabolism values above or on the upper limit of normal. According to Grafe (11) and Eberstadt (12) in posthemorrhagic conditions there is a difference in the respiratory exchange depending on the state of the bone marrow. Anemic animals with normal bone marrow have shown normal metabolism and anemic animals with exhausted bone marrow have shown diminished metabolism. From these experiments Grafe concluded that active blood formation increases metabolism. Distinctly increased metabolism was found by Lukjanow (7) and Hári (8). Hári reported a 12 per cent increase after large hemorrhages in dogs. Two objections have been raised against these and similar experimental results: First that the animals were investigated too shortly after bleeding, and second, as pointed out by Plesh and Mohr (9), that the increased metabolism besides indicating a possible effect on the activity of blood-forming organs, might also arise from a more rapid respiration and heart rate.

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The results given by Hári, Lukjanow and Grafe have been partially confirmed by the experiments of Tompkins, Brittingham and Drinker. The latter authors found that blood transfusion in anemic states caused a diminution of metabolism, diminution of pulse and respiratory activity, a drop in temperature if it had previously been elevated, a rise in the red count and hemoglobin content. The effects were not due to a decrease in muscular activity, because the lowering of metabolism began some days after the transfusion. Therefore, they conclude that in anemic animals in addition to the increase in metabolism due to increased respiratory movement and heart activity there is evidently some type of stimulation of the body cells in general which increases metabolism.

The rôle of this stimulating factor was a primary concern in our experiments. We have sought further evidence of the existence of some kind of a stimulating factor for blood cell formation in protein-free extracts of liver substance by making simultaneous respiratory exchange determinations in addition to the blood investigations during the recovery of anemic animals.

In a second series of experiments the influence of a protein-free spleen extract (spleen Extract III, *b* prepared as well as the liver extract by Dr. Jobling of Columbia University) upon blood regeneration and respiratory exchange was studied.

II. Method.

The lightly ether anesthetised animals were bled either by heart puncture or from the carotid artery. Six-tenths of the whole blood was taken when possible. The total volume of blood was computed as 5 per cent of the body weight. This computation, however, does not give the same value for the blood volume in lean and in fat animals.

The extracts were freshly made by Dr. Jobling immediately before starting the experiments. A fresh 1 per cent solution was prepared each week from the dried extract. This was made up with sterile salt solution and kept in the ice box. 2 cc. of the solution (20 mg. dried substance) were given intraperitoneally to each animal on each Monday, Wednesday and Saturday. Measurements of the respiratory exchange were made each Tuesday and Friday.

Blood examinations (blood counts Hb. determination, reticulated cells, smears) were made before and after bleeding and once each week on the same day until complete recovery or death. A Leitz counting chamber and Sahli's hemoglobinometer were used.

At the end of the experiments the animals were sacrificed and the tissues examined histologically.

The respiratory exchange apparatus used was a modified Haldane. Each respiratory exchange measurement covered a 2 hour period. The animals were

TABLE I.

	Rabbit No.	Before bleeding. Percentages of						1 wk. after bleeding. Percentages of						2 wks. after bleeding. Percentages of						3 wks. after bleeding. Percentages of					
		Total white cells	Neutro.	Eosino.	Baso.	Mono.	Lympho.	Total white cells	Neutro.	Eosino.	Baso.	Mono.	Lympho.	Total white cells	Neutro.	Eosino.	Baso.	Mono.	Lympho.	Total white cells	Neutro.	Eosino.	Baso.	Mono.	Lympho.
Control animals (anemic)	1	10,000	20	1.60	.4	15	63	6,400	23	0.5	1	4.5	71	8,100	20	2	—	7	71	10,200	47	—	3	9	41
	2	8,200	32	.4	3	2	56.6	6,800	29	3	2	8	58	8,000	38	3	.1	10	51	9,600	24	0.5	2	8.5	65
	3	12,200	24	2	—	14	60	10,200						12,400						14,200	34	1	3	12	52
Animals treated with liver extract (anemic)	4	7,900	10	4	—	7	70	8,800	60	2	—	8	30	9,600	36	1	2	11	50	12,600	29	—	2	6	63
	5	10,200	22	.1	1	5.6	70	10,200	45	1	2	10	42	9,000	41	2	3	15	39	8,600	52	—	1	5	42
	6	11,600	29	1	.4	16	50	20,000						10,400						18,000	38	2	3	10	47
Animals treated with spleen extract (Anemic)	7	10,600	28	1	2	20	49	9,800	16	8	2	12	62	11,200	5	4	1	7	83	11,600	68		—	14	72
	8	14,660	21	2	1	6	70	7,800	63	1	1	4	86	14,200	12	22	—	8	58	10,800	17	5	4	4	70
	9	5,800	39	1	2	15	52	12,600						8,000						10,400	19	6	2	12	61
(Normal)	10	12,200	32	2	3	14	49													11,800	25	4	6	15	50
	11	14,200	28	4	3	17	48													12,200	10	6	—	8	76

TABLE II.
Reticulated Cells.

	Rabbit No.	Before bleeding	1 wk. after bleeding	2 wks. after bleeding	3 wks. after bleeding
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Control animals (anemic)	1	1.0	7.2	9.6	6.9
	2	3.0	3.2	9.8	3.9
	3	1.4	4.0	16.0	3.0
Animals treated with liver extract (anemic)	4	1.4	15.0	10.0	3.7
	5	1.7	9.0	3.0	0.6
	6	1.8	11.2	9.2	0.4
Animals treated with spleen ex- tract (Anemic)	7	0.9	3.7	2.6	3.5
	8	0.8	3.5	1.0	4.0
	9	1.1	3.0	3.0	4.0
(Normal)	10	1.7	—	—	1.2
	11	0.9	—	—	1.1

TABLE III.
Summary of the Respiratory Exchange Measurements.

	Average metabolism before bleeding		Average metabolism dur- ing 1st wk. after bleeding		Average metabolism dur- ing 2nd wk. after bleeding		Average metabolism dur- ing 3rd wk. after bleeding	
	Total calories in 2 hrs.	Calo- ries per kg. per hr.	Total calories in 2 hrs.	Calo- ries per kg. per hr.	Total calories in 2 hrs.	Calo- ries per kg. per hr.	Total calories in 2 hrs.	Calo- ries per kg. per hr.
Group I Control anemic animals un- treated (3 cases)	15.27	3.06	12.88	2.64	13.53	2.71	13.84	2.71
Group II Anemic animals treated with liver extract (3 cases)	13.80	2.54	13.80	2.68	14.70	2.99	14.03	2.85
Group III Anemic animals treated with spleen extract (3 cases)	12.73	2.73	12.27	2.67	12.93	2.73	13.10	2.68
Group IV Normal animals treated with liver extract (2 cases)	13.10	2.25	15.03*	2.56	15.67	2.60	14.89	2.51
Group V Normal animals treated with spleen extract (2 cases)	12.41	2.91	12.39	2.80	13.13	2.97	11.53	2.62

* One animal pregnant.

fed for the last time 18 hours before the experiment. The standard diet (alfalfa hay, oats, with carrots or greens once a week) used in the laboratories of Montefiore Hospital was offered to the animals in equal amounts.

III. Presentation of Data.

Respiratory exchange measurements have been made on 9 anemic and 4 normal animals. For convenience of description the experi-

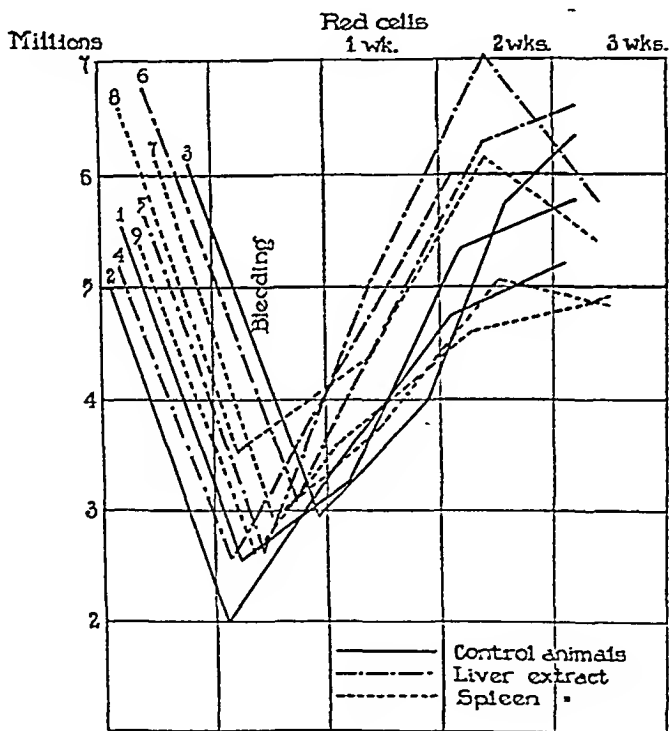


CHART 1.

ments have been grouped as follows: Group I comprises 3 anemic animals untreated and serving as controls; Group II includes 3 anemic animals treated with liver extract; Group III includes 3 anemic animals treated with spleen extract; Group IV includes 2 normal animals treated with liver extract; and Group V includes 2 normal animals treated with spleen extract.

A general summary of all the animals is given in Table III, in which the figures on heat production before bleeding represent the averages of 2 weeks readings while those after the bleedings represent the average weekly readings. The respiratory exchange measurements are also given in Charts 3 and 4. Chart 3 gives the data of the anemic

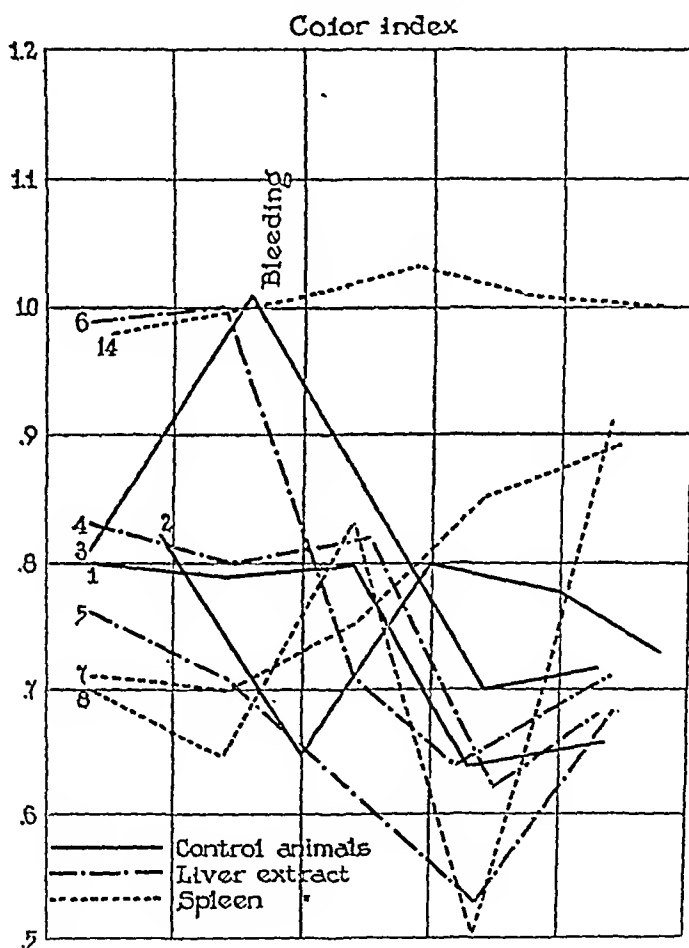


CHART 2.

animals and Chart 4 those of the normal animals. The number of red cells and color index of the anemic animals are given in Charts 1 and 2. The quantitative and qualitative changes in the white blood elements are shown in Table I. The figures of the reticulated cells of the anemic animals are contained in Table II.

IV. DISCUSSION.

A study of the respiratory exchange measurements of the five groups of experiments (see Table III) shows only slight differences in the total metabolism. There is a slight increase in the group of anemic animals treated with liver extract, while in those treated with spleen extract no notable change occurred.

The blood pictures, hematological and histological findings in all groups were approximately the same as those observed on a larger series of anemic rabbits, when we studied the question of blood regeneration under different conditions.

The anemic animals treated with liver extract showed a more rapid numerical restoration of the red blood cells, more reticulated cells, a lower color index and more prominent lymphoid tissue throughout the body than did the controls or those treated with spleen extract.

The slight increase in heat production observed during the period of blood regeneration in animals treated with liver extract coincides with and may be correlated with the hematological findings and suggests that liver extract exerts a stimulating effect on blood cell formation (Fig. 1). As to the nature or mode of action of this liver extract nothing definite can be said. In this connection we may recall that Joannovics and Pick (14) in 1909 obtained an alcohol and acetone-soluble hemolytic substance from the liver of animals treated with toluylenediamine and we know from experiments of others as well as from our own that the destruction products of the red cells do accelerate red blood cell formation. It may be possible too that in the liver extract a specific substance is present which stimulates blood cell regeneration.

Anemic animals treated with spleen extract showed that the numerical restoration of the red blood cells was not complete, while the heat production measurements indicated a possible slight decrease. Perhaps the spleen produces a substance inhibiting blood regeneration (see Chart 1 and Fig. 2), and in the meantime lowering the general metabolism (see Charts 3 and 4).

Richet (18) published in 1912 a paper in which he stated that splenectomized dogs need more food to keep their body weight in balance than normal animals. It is said that after splenectomy there

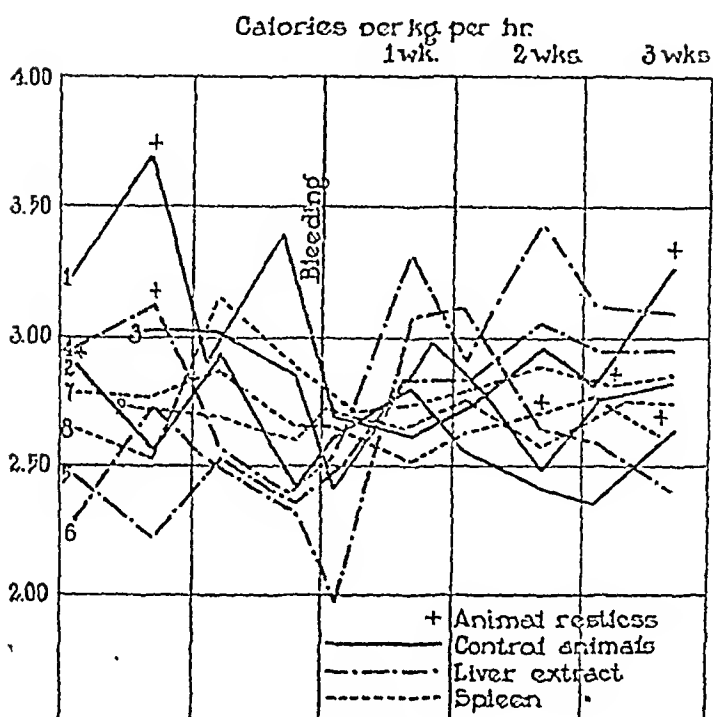
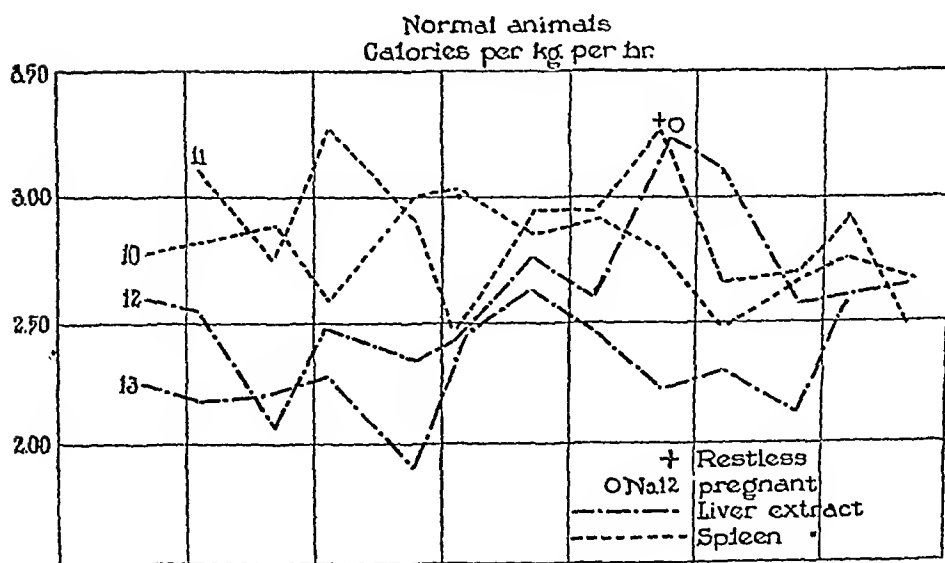


CHART 3.



Treatment

CHART 4.

is a hyperplasia of the thyroid gland. In cases of goiter, enlargement and fibrosis of the spleen is frequently seen. The possible antagonistic relation between the spleen and thyroid gland was first investigated by Asher and Streuli (19). They reported that after splenectomy, rats with intact thyroids are less resistant toward diminished partial pressure of oxygen than normal rats. After thyroidectomy, on the contrary, the rats became more resistant against asphyxia. After splenectomy in rats the basal metabolism of animals was found to be increased by Danoff (20), Asher and Hauri (21). Marine and Baumann (22) found no significant change in rabbits. We produced some sort of a "hyper-splenia" with a protein-free spleen extract. We observed a fall in the basal metabolism both in normal and anemic animals treated with spleen extract. The blood cell regeneration was retarded also in these cases. 1 year after I finished my experiments M. and A. Lefikowitz (23) published their results, obtained with extracts from the spleen of anemic animals. This extract inhibited moderately red cell formation. The temperature curves of the several groups of experiments remain normal throughout the period of observation. The thyroid glands were more vascular and histological examination showed them to be slightly hypertrophic. This was undoubtedly true in those animals treated with liver extract. Such evidence of increased functional activity of the thyroid has long been noted in anemias, both in man and animals, and was formerly used in support of the view that the thyroid was directly concerned with blood formation (Fig. 3).

VI. SUMMARY AND CONCLUSION.

1. Respiratory exchange measurements may be used for gaining further evidence concerning the body changes during anemia, and are complementary to the hematological and histological data.
2. The heat production during anemia was slightly decreased. At the 3rd week it began to rise.
3. A protein-free liver extract has accelerated blood regeneration and at the same time increased the respiratory exchange of anemic animals.
4. A protein-free spleen extract has distinctly lowered the respiratory exchange of normal animals. The metabolism of anemic animals thus treated was practically the same as before bleeding and treatment.

The recovery of the spleen extract-treated animals was not complete during the period of observation. This extract may have been somewhat toxic. In spite of this disturbing factor we are not inclined to accept the view of C. D. and E. W. Leake (15) and Thalhimer (16) that the spleen takes part in the stimulating effect upon blood regeneration when given in combination with red bone marrow by mouth.

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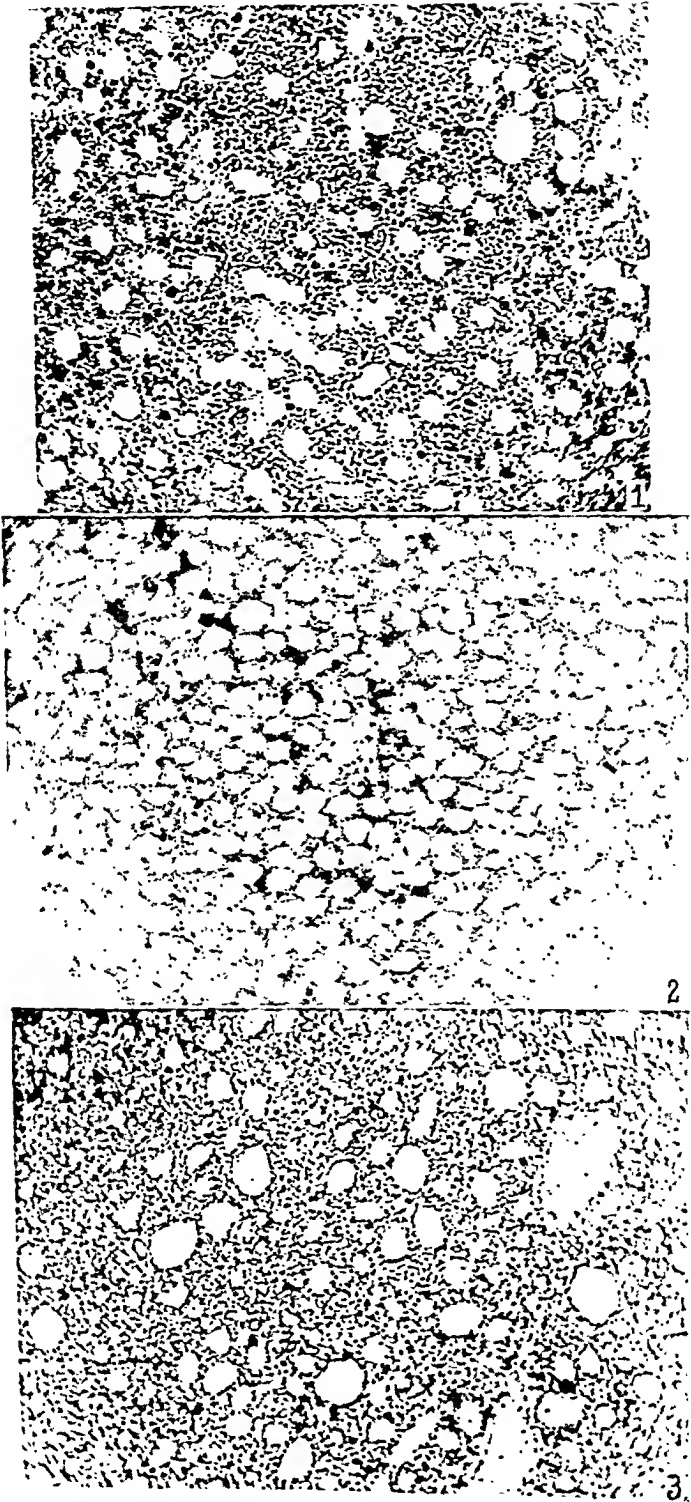
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EXPLANATION OF PLATE 30.

FIG. 1. Bone marrow of Rabbit 5. Anemic animal treated with liver extract. 4 weeks after bleeding.

FIG. 2. Bone marrow of Rabbit 7. Anemic animal treated with spleen extract. 4 weeks after bleeding.

FIG. 3. Thyroid gland of Rabbit 5. Anemic animal treated with liver extract. 4 weeks after bleeding.



VITAMIN A DEFICIENCY AND METAPLASIA.

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INTRODUCTION.

Mori (1) was the first to draw attention to the change which occurs in the lining epithelium of the larynx, trachea, and ducts of the Meibomian, submaxillary, sublingual, and parotid glands of rats that have been fed a diet deficient in the fat-soluble vitamins. He referred to the change as keratinization, and, as in the case of xerophthalmia (2), attributed it to the drying of the epithelium (xerosis) due to the hypofunction of the glands whose secretion keeps these epithelial surfaces moist. The impaired activity of these glands he considered to be due to the pathological changes (atrophy and degeneration) induced in them by the vitamin deficiency.

Wolbach and Howe (3, 4) recently confirmed the findings of Mori (1), examined many more organs and tissues than did Mori, and found the changes in some of them, as well as in some (pancreatic duct and renal pelvis) in which Mori had not found any. Wolbach and Howe referred to the change as a metaplasia of the cylindrical or cuboidal or transitional type of epithelium to the squamous keratinizing type, and attributed to the fat-soluble vitamin deficiency the direct specific cause of the metaplastic changes. They also found the other morphological changes described by Mori in paraocular, submaxillary, and other serous and mucous glands, but did not consider that the lack of the secretions of these glands induced the metaplasia.

The diets employed by Mori (1) and by Wolbach and Howe (4), although referred to by them as deficient in fat-soluble A, were actually deficient in vitamin A (antiophthalmic, growth-promoting), vitamin C (antiscorbutic), and vitamin D (antirachitic), but this fact was recognized by the authors.

Since it has been shown that xerophthalmia will develop as a result of a dietary deficiency of vitamin A alone, and since the diets employed by Mori and by Wolbach and Howe were deficient in several vitamins, it seemed of interest to us to determine whether a single vitamin deficiency (vitamin A) could also induce the metaplasia and other changes in glandular organs, that have been described by these investigators.

EXPERIMENTS.

The Animals.

A total of 63 piebald black and white rats bred in this institution were used in this investigation. The sex, number of animals in every group, initial, maximal, and final weights, and length of time on the special diets are given in Tables I to III. Most of the rats were from 28 to 35 days old at the beginning of the experimental period. The animals were kept in individual wire mesh cages in a well ventilated room protected from direct sunlight. Representatives from every litter were put into three groups. Group I received a diet deficient in vitamins A and D (Diet -A-D), but complete in all other respects; Group II received a diet deficient in vitamin A alone (Diet -A); and Group III received a diet complete in every respect (Diet +C. L. O.).

The Diets.

Group I.—(Diet -A-D.) This diet is deficient only in vitamins A and D, and consists of the following:

Inactivated* technical casein (Merck).....	20 gm.
Corn-starch.....	50 gm.
Cottonseed oil.....	15 gm.
Salt mixture, McCollum No. 185 (6).....	5 gm.
Vegex (Marmite) (for vitamin B).....	5 gm.
Decitrated lemon juice (for vitamin C).....	5 cc.
Distilled water.....	50 cc.

* By heating and aeration (Goldblatt and Moritz (5)).

Group II.—(Diet -A.) This diet is deficient only in vitamin A. The ingredients of the diet are exactly the same as those of Group I, but in order to supply the antirachitic factor, the cottonseed oil was irradiated by a mercury vapor quartz lamp in a manner described before (Goldblatt and Moritz (7)).

Group III —(Diet +C. L. O.) The diet is the same as that of Group I, but in order to supply the missing vitamins A and D, and to make the diet complete in every respect, 7 drops (about 150 mg.) of cod liver oil (Harris) were administered daily by mouth to every rat in this group.

The rats of Groups I and II ate from 10 to 20 gm. daily in the beginning, and from 7 to 15 gm. later, while those in Group III ate from 15 to 25 gm. daily throughout the experimental period. The animals were sacrificed after they had been on these diets for varying periods. (See Tables I to III.)

Autopsy Findings.

The only gross morbid changes seen were occasional lobular pneumonia in the three groups, abscesses in the base of the tongue in practically every rat of Groups I and II in which this organ was examined, xerophthalmia in some of the rats of Groups I and II, an occasional abscess in the submaxillary gland in Groups I and II, and in a few instances, in Groups I and II, dilated ureters or dilated external bile ducts.

The tongue abscesses are of special interest. They have been described by Sherman and Munsell (8) who found them in 76 per cent of their rats on a diet deficient in the fat-soluble organic factor. Wolbach and Howe do not speak of abscess, but of cysts, and they say that: "Infection complicates the picture as it causes complete destruction of the epithelium lining cysts and repair by granulation tissue accompanied by foreign body reactions to the retained keratinized cells." The abscesses are probably painful and, more than the loss of sense of smell, mentioned by Wolbach and Howe, may explain, at least in part, the suddenness with which many of the rats cease to eat and in consequence lose much weight. Smears were made of the pus from these abscesses; organisms were always found, and practically always they were a mixture of Gram-positive diplococci and Gram-negative bacilli, some free, others inside pus cells. Occasionally, the only organisms were Gram-positive diplococci. Cultures in broth and on solid media showed the same organisms, but they have not yet been definitely identified. In no instance were abscesses of the tongue or submaxillary glands found in the rats of Group III (+ C. L. O.).

Histological Findings.

The tissues were either fixed in Zenker's fluid and stained with eosin and methylene blue, or fixed in formalin and stained with hematoxylin and eosin. In many instances half of an organ was treated by one

and the remainder by the other method. Both were very satisfactory for the recognition of metaplastic changes. Sections were made at various levels of an organ. The stomach was not investigated histologically. Keratinization of the cornea and conjunctiva alone was not considered a positive result.

In the case of about two-thirds of the rats of every group all the organs investigated by Wolbach and Howe were examined histologically, but in the remainder, only nasal passages, larynx, trachea, bronchi, lungs, and tongue were investigated microscopically. For our purpose this was deemed sufficient because, in those rats of Groups I and II in which all organs were examined, no metaplastic changes were seen in any other organ when there were none in the accessory salivary glands of the tongue and in the respiratory tract. Wolbach and Howe also state that the changes practically always appear first in the respiratory tract. Besides, in no rat of Group III (normal diet) was squamous keratinizing epithelium found in an abnormal situation (see Table III). Since we were using metaplasia as a morphological indicator, and since a positive finding in a single organ was adequate for our purpose, the results still further justify the procedure because the metaplastic changes were found in some part of the respiratory tract and in the accessory salivary glands of the base of the tongue in practically all the rats in Groups I and II (see Tables I and II). Similar changes were found in fewer instances in the ducts of paraocular, submaxillary, sublingual, and parotid glands, and in renal pelvis, ureter, and bladder. The manifestations were similar to those described by Mori and by Wolbach and Howe; they varied from small isolated nests to complete transformation of the epithelium in the sections examined, and appeared with or without accompanying inflammation. When inflammation was present, the metaplastic changes were usually more severe. Even in the case of the accessory salivary glands of the base of the tongue, often, in the one organ, there were large cysts lined completely, or in part, by squamous epithelium, filled with keratohyaline material, and without any signs of inflammation, while in another portion there was a large abscess lined partly, sometimes completely, by squamous keratinizing epithelium. It would appear therefore, that the inflammatory process occurs coincident with or subsequent to the metaplasia, and is not responsible for the initiation

of the changes. This is also the view of Mori and of Wolbach and Howe. Atrophy and degeneration of the epithelium of the testicular tubules and of the acini of the salivary and paraocular glands were a common finding in Groups I and II, but did not occur in Group III. This agrees with the findings of Mori and of Wolbach and Howe.

TABLE I.
Group I (Diet -A-D).

Number of rat	Sex	Days on diet	Weight			Metaplasia	Remarks
			Initial	Maximal	Final		
			gm.	gm.	gm.		
1	M.	137	52	174	149	Absent	Bronchopneumonia
2	M.	133	45	167	149	Present	Xerophthalmia
3	M.	140	45	162	142	Present	Xerophthalmia
4	M.	115	50	147	125	Present	Dilated ureters and bile ducts; xerophthalmia
5	M.	95	57	108	94	Absent	Xerophthalmia
6	M.	95	44	120	101	Present	Bronchopneumonia
7	M.	95	46	70	63	Present	Xerophthalmia; bronchopneumonia
8	M.	88	47	98	92	Present	—
9	M.	79	38	83	73	Present	—
10	M.	98	60	195	188	Present	Abscess of tongue
11	M.	70	55	136	110	Present	Abscess of tongue
12	M.	72	45	151	130	Present	Xerophthalmia; abscess of tongue
13	M.	77	40	142	115	Present	Abscess of tongue and submaxillary glands
14	M.	70	43	155	121	Present	Xerophthalmia; abscess of tongue
15	M.	83	55	110	95	Present	Abscess of tongue
16	F.	95	46	98	95	Present	—
17	F.	95	51	84	80	Present	—
18	F.	95	46	90	75	Present	Bronchopneumonia
19	F.	89	40	82	65	Present	—
20	F.	90	38	79	73	Present	—
21	F.	85	36	66	56	Present	Bronchopneumonia
22	F.	98	47	137	135	Absent	—
23	F.	98	51	147	145	Present	Xerophthalmia; abscess of tongue
24	F.	70	45	121	100	Present	Xerophthalmia; abscess of tongue
25	F.	70	35	113	100	Present	Abscess of tongue
26	F.	84	35	117	75	Present	Xerophthalmia

TABLE II.
Group II (Diet -A).

Number of rat	Sex	Days on diet	Weight			Metaplasia	Remarks
			Initial	Maxi- mal	Final		
			gm.	gm.	gm.		
27	M.	101	44	166	120	Present	Xerophthalmia; abscess of submaxillary glands
28	M.	95	53	187	187	Present	—
29	M.	74	51	125	88	Present	Enteritis
30	M.	116	61	153	132	Present	—
31	M.	78	55	166	125	Present	Abscess of tongue
32	M.	91	35	181	150	Present	Abscess of tongue
33	M.	98	40	188	148	Present	Abscess of tongue
34	M.	63	42	135	113	Present	Abscess of tongue
35	M.	71	42	148	112	Present	Abscess of tongue
36	M.	56	55	120	102	Present	Xerophthalmia; abscess of tongue
37	F.	74	47	150	123	Present	Xerophthalmia; bronchopneumonia
38	F.	95	52	150	148	Absent	Xerophthalmia
39	F.	98	50	144	141	Present	Dilated ureters
40	F.	94	55	135	135	Present	Abscess of tongue
41	F.	78	48	129	97	Present	Abscess of tongue
42	F.	78	45	128	91	Present	Abscess of tongue
43	F.	91	33	128	97	Present	Abscess of tongue and submaxillary glands
44	F.	98	38	145	110	Present	Abscess of tongue; xerophthalmia

TABLE III.
Group III (Diet +C. L. O.).

Number of rat	Sex	Days on diet	Weight			Metaplasia	Remarks.
			Initial	Maxi- mal	Final		
			gm.	gm.	gm.		
45	M.	144	52	240	208	Absent	Bronchopneumonia
46	M.	137	44	233	228	Absent	—
47	M.	95	48	228	170	Absent	—
48	M.	95	38	216	216	Absent	—
49	M.	95	40	253	253	Absent	—
50	M.	77	39	258	258	Absent	—
51	M.	77	37	241	241	Absent	—
52	M.	77	37	212	212	Absent	—
53	M.	56	50	168	168	Absent	—
54*	M.	77 + 11	45	250	146	Absent	—
55*	M.	56 + 16	43	222	124	Absent	—
56*	M.	77 + 9	45	240	147	Absent	—
57*	F.	56 + 16	40	150	94	Absent	—
58*	F.	77 + 11	45	280	166	Absent	—
59*	F.	77 + 9	40	150	96	Absent	—
60	F.	95	45	191	191	Absent	—
61	F.	95	42	174	174	Absent	—
62	F.	95	42	160	160	Absent	Bronchopneumonia
63	F.	77	35	170	170	Absent	—

* These rats were on diet (+C. L. O.) for the first period indicated in the column headed "Days on diet" and were deprived of all food except a mixture containing vitamins A, B, C, and D for the second period indicated in the same column.

TABLE IV.

	Number of rats in group	Number of rats showing epithelial metaplasia
Group I (Diet -A - D).....	26	23
Group II (Diet -A).....	18	17
Group III (Diet +C.L.O.).....	19	0

Special Note.

Since, as is usual, many of the rats on $-A$ and $-A-D$ diets ate but little during the last week or two of their lives and often lost much weight, it was conceivable that the lack of food in itself might play some part in initiating the metaplasia. An experiment was therefore performed to test the effect of deprivation of food. Included in Group III (Diet + C. L. O.) are six rats (Nos. 54, 55, 56, 57, 58, and 59) which had been receiving Diet + C. L. O. for from 56 to 77 days and after that were deprived of food but were given an adequate amount of water and of vitamins A, B, C, and D in the form of a mixture of cod liver oil (about 150 mg.), Marmite (0.5 gm.), and decitrated lemon juice (0.5 cc.), which was administered daily by mouth. In no instance were metaplastic changes seen in any of the organs of these six rats, but atrophy of most tissues, even of the lining epithelium of the trachea and bronchi, was in most instances very great. Abscess of the tongue did not occur in these rats.

SUMMARY.

Of nineteen rats on a complete diet (Group III, Diet + C. L. O.), none showed foci of squamous keratinizing epithelium in abnormal situations.

Of twenty-six rats on a diet deficient in vitamins A and D (Group I, Diet $-A-D$), twenty-three showed metaplastic changes of varying degree in one or more organs; the metaplasia was of columnar, cuboidal, and transitional epithelium to the squamous keratinizing type.

Of eighteen rats on a diet deficient in vitamin A alone (Group II, Diet $-A$), seventeen showed epithelial metaplasia similar to that of Group I in one or more organs.

In Groups I (Diet $-A-D$) and II (Diet $-A$) the changes occurred in one or more of the following organs: trachea, large bronchi, small bronchi or bronchioles in lung, posterior nares, accessory salivary glands of base of tongue, paraocular, submaxillary, sublingual, and parotid glands, renal pelvis, ureter, and bladder. The metaplastic changes were as extensive in the rats of Group II on a diet deficient in vitamin A alone as in those of Group I which received a diet deficient in vitamins A and D.

CONCLUSION.

1. A dietary deficiency of vitamin A alone is adequate to induce metaplasia of columnar, cuboidal, and transitional epithelia to the squamous, keratinizing type in some organs.

2. Epithelial metaplasia to the squamous keratinizing type is of such frequency in young rats kept on a diet deficient in vitamin A for a long period of time (10 weeks or longer) as to constitute a good morphological indicator of the deficiency of this vitamin in the diet.

3. The metaplastic changes are as frequent and as great in rats on a diet deficient in vitamin A alone as in those on a diet deficient in vitamins A and D.

4. Inadequate food, provided the vitamins are supplied, does not induce the metaplasia.

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THE OXYGEN CONTENT OF THE VENOUS BLOOD OF THE DOG AFTER UPPER GASTROINTESTINAL TRACT OBSTRUCTION.

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The color of the venous blood of the dog after gastrointestinal tract obstruction is a good index of the degree of toxemia. With the characteristic rise in non-protein nitrogen the blood becomes increasingly darker in color. Cyanosis has also been recognized as one of the cardinal clinical signs of such toxemia in man. The change of color is due to the presence of an increased amount of reduced hemoglobin. To measure this change, determinations of the oxygen content and oxygen capacity of the venous blood of the dog have been made after experimental obstruction at different levels.

Method.

All operations were done under ether anesthesia with aseptic technique. Obstruction of the cardia and pylorus was accomplished by ligating with heavy tape. The intestine was obstructed by section of the upper end of the jejunum and inversion of the cut ends.

Blood for chemical analysis was withdrawn from the jugular vein. Specimens for the determination of oxygen content were collected under oil. The total oxygen capacity was determined on venous blood after thorough shaking with air. The oxygen saturation of arterial blood was determined on samples collected under oil from the femoral artery. All oxygen determinations were made by Van Slyke's technique (1). The non-protein nitrogen was determined by the method of Folin and Wu (2), and the chlorides on the tungstic acid filtrate after the method suggested by Gettler (3).

EXPERIMENTAL OBSERVATIONS.

After obstruction of the cardiac end of the stomach there is a rapid and marked fall in the oxygen content of the venous blood (Table I). Thus the oxygen saturation of the blood of Dog 2 before

operation was 54 per cent and on the 3rd day after obstruction was only 3 per cent. The findings after pyloric obstruction (Table II) are equally marked. There is also a decrease in oxygen saturation after intestinal obstruction although this is not so striking as with obstruction at higher levels.

TABLE I.
Oxygen Saturation of Venous Blood after Cardiac Obstruction.

Dog No.	Day after	Blood				Remarks
		Hema- tOCRIT reading (per cent of cells)	Oxygen saturation of venous blood	Chlorides	Non-pro- tein nitrogen	
		<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	
1	0		56		36.1	
	1		9		41.6	
	2		14	395	89.8	O ₂ saturation of arterial blood, 95 per cent
2	0	49	54	430	47.6	
	1	50	26	460	46.0	
	2	51	46	430	65.5	
	3	54	3	460	101.0	
	3	35	56	600		Immediately after receiving 50 cc. 10 per cent NaCl
	4	42	35	590	107.0	
3	0	51	57	460	44.4	
	1	55	33	410	60.0	
	2	60	30	390	45.6	
	3	54	33	370	54.5	
	4	52	41	360	118.0	
	5	52	7	350	183.0	
4	0	54	53	450	25.9	
	1	62	50	380	81.0	
	2	60	19	370	114.0	O ₂ saturation of arterial blood, 93 per cent

DISCUSSION.

The average oxygen saturation of the venous blood of 12 dogs before operation was 60.8 per cent and only 16.8 per cent at the end

of the experiment. This marked decrease in oxygen saturation must be due to incomplete oxidation in the lungs or to an increased reduction of normally saturated hemoglobin in the capillaries. The oxygen content of the arterial blood was determined a number of

TABLE II.

Oxygen Saturation of Venous Blood after Pyloric Obstruction.

Dog No.	Day after operation	Blood				Remarks
		Hemato- crit reading (per cent of cells)	Oxygen saturation of venous blood	Chlorides	Non-pro- tein nitrogen	
		<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	
5	0	55	50	475	40.0	
	1	60	14	440	71.3	
	2	53	5	390	198.0	
6	0	45	63	450	28.0	
	1	58	21	430	63.5	
	2		9	410	133.0	Obstruction released
	3	48	22	390	205.0	
	4	42	17	390	147.0	
	5		26	380		
7	0	48	46	450	36.1	
	1	53	8	440	27.0	O ₂ saturation arterial blood, 100 per cent
	2	54	37	410	48.7	
	3	61	22	340	167.0	
8	0	53	88	460	46.9	O ₂ saturation arterial blood, 95 per cent
	1	57	16	400	40.0	
	2	60	50	340	60.5	O ₂ saturation arterial blood, 99 per cent
	3	52	10	280		O ₂ saturation arterial blood. 88 per cent

times when the oxygen content of the venous blood was very low and found always within normal limits, showing there is no disturbance of oxidation in the lungs.

The increased deoxidation in the capillaries may be due to a slower

flow than normal. Such occurs after exercise if not compensated for by an increased rate of flow or in heart disease after decompensation (4). After upper gastrointestinal tract obstruction the viscosity of the blood is much increased (5), tending to slow the flow. Dehydration may also be a factor although the relative cell mass is little

TABLE III.

Oxygen Saturation of Venous Blood after Intestinal Obstruction.

Dog No.	Day after operation	Blood			
		Hematocrit reading (per cent of cells)	Oxygen saturation of venous blood	Chlorides	Non-protein nitrogen
		<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
9	0	44	41	460	26.5
	1	51	57	460	33.0
	2	54	26	390	116.0
10	1	46	71	430	
	2	47	36	390	25.7
	3	49	30	300	31.9
	4	46	27	250	65.0
11	1	57	84	440	34.5
	3	60	62	350	41.4
	4	58	49	320	63.0
	5	58	61	300	122.0
	6	53	62	270	105.0
	8	55	60	235	126.0
	10	55	50	200	179.0
	12	53	23	190	234.0
	13	53	11	170	263.0
12	1	51	66	450	46.8
	2	65	32	360	86.3

changed as shown by hematocrit readings. Keith (6) studied the oxygen saturation of the venous blood after experimental dehydration and found no marked decrease even with a very great increase in viscosity. The rate of flow through the capillary bed is much slowed also in shock.

It seems probable that each of the factors enumerated may play

a part in the decreased oxygen saturation. It is also possible that there may be present in the blood stream and body tissues active reducing bodies which are wholly or in part responsible for the greatly increased deoxidation. Thunberg (7) has demonstrated such reducing bodies in other conditions by their action on methylene blue. These, he thinks, are intermediate products of metabolism. In upper gastrointestinal tract obstruction there is much evidence to suggest the view that such reducing bodies are present. This evidence will be discussed fully in a later paper.

SUMMARY AND CONCLUSIONS.

There is a rapid fall in the oxygen content of the venous blood of the dog after upper gastrointestinal tract obstruction. This increased deoxidation is probably due to a combination of several factors.

There is much evidence to suggest that active reducing bodies are present in the blood.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

IV. EVIDENCE FOR THE PENETRATION OF HIGH FREQUENCY CURRENTS THROUGH THE LIVING BODY.

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INTRODUCTION.

We have previously intimated that many of the statements which are to be found in the clinical, and more particularly in the commercial, literature, concerning the passage of high frequency currents through the animal body, are fallacious. The source of these fallacies appears to us to depend first upon certain misconceptions regarding the nature of the currents, and secondly upon arguments from analogy derived from experiments done on artificial systems. There is *a priori* no objection to this type of experiment. Indeed the *in vitro* experiment offers many advantages over the complicated conditions prevailing in the cadaver or the living animal body. Yet, as we have said before, the assumption that the intact animal will behave in the same manner as a sausage or a piece of meat is obviously unsound and must lead to confusion and error.

The transportation of alternating currents of high frequency and the laws governing the transfer are essentially the same as in the case of low frequency alternating currents or continuous currents. Many of these laws must, however, be modified in order to apply to those conditions peculiar to the rapidly oscillating cycles of the diathermy current. It is not intended to enumerate all the various influences which render the modifications necessary, although a knowledge of them is essential to predict the course which the current will follow in passing between two electrodes. Knowledge of the laws has led to the discovery and utilization of high frequency currents in wireless telegraphy and telephony. An immense amount of expert labor has been expended on this subject. It is futile, therefore, for electro-

theraputists to draw sweeping conclusions from haphazard *in vitro* experiments.

Three questions which have mainly concerned those interested in the study of high frequency currents in relation to diathermy are: (1) Does the current take the shortest path between electrodes rather than the path of least resistance? (2) Is heat production greatest at a point midway between electrodes? (3) Does the so called "skin effect" determine the path of the current in its passage between electrodes? These questions will be considered in this paper.

The statement is frequently encountered that heat is generated first in the interior of the body between the two electrodes, then at the surface. If the electrodes are of equal shape and area, the hottest point is said to be midway between them; if unequal, the hottest point will be nearer the smaller of the two. By varying the relative areas of electrode surfaces it is held that the localization and depth of heat can be controlled more or less accurately. Such statements are based on demonstrations made on the cooking of meat or the coagulation of egg white. They seldom, if ever, take into account the fact that the surfaces of these media will, of course, cool more rapidly than their centers, and that, though surface cooling may be true of artificial systems, it need not necessarily hold for the living body. Indeed, it can be categorically stated that it is not true for the living body and that heating with diathermy occurs from without inward. There is, as will be presently shown, a definite heat gradient from subcutaneous tissue to lung. The skin, of course, will cool more rapidly than the subcutaneous tissues.

Even in an artificial system Bettman and Crohn (1) have shown that coagulation of egg albumin "occurred first near each electrode, then spread toward the center in gradually enlarging arcs approaching each other" and finally meeting in the center to include all the medium between the two electrodes. They state that "this experiment was repeated innumerable times with variations in the amount of current and the size of the electrodes, as well as the speed in which the current increased, with identical results. It was found that it did not make any difference whatever how quickly or slowly the amount of current was increased. This is in contradiction to numerous assertions that, in order to get so called central heating, the current must be increased slowly." They were "never able to obtain a coagulation of the albumin first in the center."

It seems apparent that the localization of maximum heat in any medium will be determined by several quite independent conditions. In any attempt to generalize or predict about heat production all of these must be borne in mind and appropriate weight given to each. First must be considered the specific resistances of the conductor which in a heterogeneous medium will, of course, be variable. Secondly, we must know the path of the current through this conductor, whether in straight, converging, or diverging lines. And, thirdly, we must not neglect the problem of cooling, which will occur at different rates in different parts of the medium and will thus variously influence the elevation of temperature.

In spite of the fact that we believe that experiments with artificial systems may be misleading, especially if uncritically applied to the living body, we have performed several such experiments simply for the purpose of exemplifying some of the fundamental principles which govern the passage of high frequency alternating currents.

EXPERIMENTAL.

Part I. Experiments on Non-Living Material.

I. Does the Current Take the Shortest Path or the Path of Least Resistance?

Experiment 1.—A basin was filled with an $M/20$ solution of NaCl . An arch made by bending a strip of lead tin 20.5 cm. long by 8 cm. wide was placed in the dish in such a manner that the parallel arms of the arch were immersed in the solution while the bent portion projected above the surface of the liquid. The parallel arms were then converted into electrodes by soldering to each a short length of copper wire which was connected to the leads of the diathermy machine.

During the passage of the current between the electrodes the rise in temperature of the salt solution was measured. The lead-tin arch was then cut and the temperature rise in the salt solution again measured. It was found that the salt solution heated over 30 times as fast when the metal arch was divided as when it was intact. This shows that the current flow had been through the metal arch, or in other words, through the longer and less resistant path. Fig. 1 shows, in diagram, the essential features of this experiment, and Table I the temperature changes observed.

Experiment 2.—An experiment with a similar object to the one just described was done by placing an agar block of relatively high ohmic resistance in a reservoir of $M/20$ saline and passing a current between electrodes immersed in the saline. Fig. 2 shows the experimental arrangement. Temperature readings were made

by thermocouples imbedded in the agar block and immersed in the salt solution in the positions indicated in the diagram. It was found that the saline heated up at a slightly higher rate than the agar. Were the current passing through the agar block, one would expect the reverse effect because the agar has a higher resistance than the saline. We can conclude, therefore, that most of the current was passing

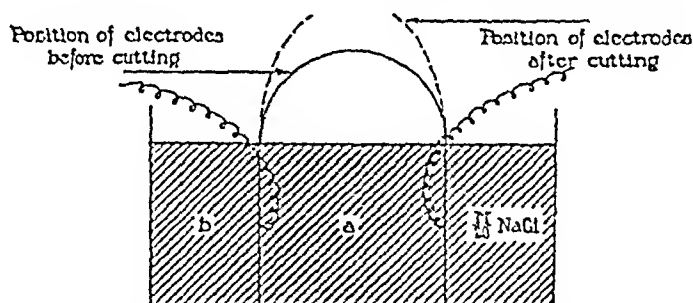


FIG. 1. Diagrammatic representation of Experiment 1. *a* and *b* indicate the position of the thermometers immersed in the salt solution. Temperature readings are shown in Columns *a* and *b* of Table I.

TABLE I.

Temperature Changes Recorded in Experiment 1.

(*a*) and (*b*) refer to the thermometers, the positions of which are shown in Fig. 1.

Position of thermometer	(<i>a</i>)	(<i>b</i>)
	°C.	°C.
Temperature elevation before cutting (current passed for 30 min.).....	.09	.06
Temperature elevation after cutting (current passed for 25 min.).....	2.35	2.33

TABLE II.

Temperature Changes Recorded in Experiment 2.

Position at which temperature was taken	Thermocouple 1	Thermocouple 2	Thermocouple 3	Thermocouple 4	Thermocouple 5	Thermocouple 6	Room temperature
	°C.	°C.	°C.	°C.	°C.	°C.	°C.
Before passage of current. . . .	18.92	19.07	19.00	19.18	19.00	19.00	21.2
After passage of current. . . .	20.58	20.97	21.43	21.80	21.98	21.92	22.4

along the longer path and the one of lower resistance. In the control experiments (Nos. 3 and 4) we found that application of the electrodes directly to the agar caused it to heat, and indeed melt, showing that it could be warmed by the passage of a current through it. The changes in temperature recorded are presented in Table II.

On the basis of observations made in these two experiments we conclude that in both metallic and electrolytic conductors the high frequency current will traverse the path of least resistance rather than the shortest path between electrodes. In making this generalization it must be remembered that there are forms of resistance peculiar to this type of current. Thus, owing to self-induction, even a thick copper wire has a high impedance to high frequency currents, and the observation that the current will pass through a short length of

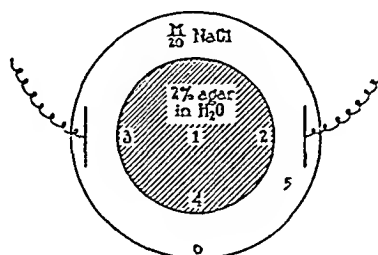


FIG. 2. Diagrammatic representation of Experiment 2. Shaded circle indicates the position of the agar block; 1, 2, 3, 4, 5, 6, the location of the thermocouples. Temperature readings are presented in Table II.

Dimensions of elec-

trodes	9 cm. X 5 cm.	Volume of agar block	1600 cc.
Distance between elec-			
trodes	19 cm.	Depth of agar block	6 cm.
Volume of saline	2000 cc.	Diameter of agar block	14 cm.
Depth of saline	6 cm.	Strength of current	700 ma.
Diameter of reservoir	25 cm.	Duration of current	1 hr., 43 min.

Position of thermocouples is indicated in Fig. 2 by the numbers 1 to 6.

sausage, rather than a long length of copper wire, is not, therefore, germane to the point under discussion.

II. Does Heating Occur First at the Center and Then at the Periphery?

We have already enumerated the various conditions which may influence the localization of heat, and we have found it necessary to consider not only specific resistance, but heat loss, also current concentration or the distribution of the lines of flow. The observations of Bettman and Crohn referred to above showed that in passing a current through an egg-agar medium coagulation occurred first at the site of the greatest current density, which, when the electrodes were

parallel, was always at the periphery or in that portion of the medium immediately approximating the electrodes.

Our experiments gave similar results. We chose for our medium a 2 per cent agar in distilled water. This could be molded into suitable forms. It offered sufficient resistance to develop heat, was a poor conductor of heat, and would cool more rapidly from its surface than its center. Did central heating actually occur before peripheral, we were dealing with an almost ideal medium on which to demonstrate it.

Experiment 3.—A cylinder was made of 2 per cent agar in distilled water. It measured 16 cm. long by 10 cm. wide. A circular lead-tin electrode 7.5 cm. in

TABLE III.
Temperature Changes Recorded in Experiment 3.

Position of thermocouple. Distance in cm. from center	5.0	2.5	0	2.5	5.0
	°C.	°C.	°C.	°C.	°C.
Before passage of current.....	21.89	21.96	22.02	21.96	21.83
After passage of current.....	25.87	24.88	24.61	24.83	25.77

Agar used, 2 per cent in distilled water.

Diameter of agar, 10 cm.

Diameter of electrodes, 7.5 cm.

Distance between electrodes, 16 cm.

Duration of passage of current, 5 minutes.

Strength of current, 1000 milliamperes.

diameter was fastened firmly to each end of the agar cylinder. A current of 1000 milliamperes was then allowed to pass between the electrodes for 5 minutes. Thermocouples were imbedded to an equal distance in the substance of the agar cylinder; one at its center and four others disposed equidistantly from the center toward the two ends. Before the current was turned on the temperature at the center was 22.02°C. at the points 2.5 cm. from the center 21.96°C., and at the ends 21.89°C. and 21.83°C., respectively. The agar cylinder was obviously cooling more at the ends than at the center. The thermocouples were then removed and reinserted immediately after the current was turned off. After 5 minutes of current flow the temperature at the center had risen 2.59°C., at the points half way between the center and the ends 2.90°C., and at the ends adjoining the electrodes 4.01°C. The actual temperature readings are given in Table III.

The conclusion to be drawn from this experiment is that in a homogeneous medium heating occurs first in those portions of the system

adjoining the electrodes. This was true here in spite of the fact that the ends of the cylinder were cooling more quickly than the center. Since the resistance of the cylinder was no greater at the ends than at the center the increased heating at the ends can only be ascribed to a concentration of current near the electrodes and to divergent lines of flow as it traversed the agar cylinder. The truth of this may be seen from the next experiment in which the lines of current flow were concentrated at the center, where maximal heating occurred.

Experiment 4.—The same agar cylinder as described in Experiment 3 was used here but it was altered by thinning its central diameter so that it finally represented two cylindrical blocks of agar 10 cm. in diameter connected by an isthmus

TABLE IV.
Temperature Changes Recorded in Experiment 4.

Position of thermocouple. Distance in cm. from center	5.0	0	5.0
	°C.	°C.	°C.
Before passage of current.....	22.39	21.57	21.06
After passage of current.....	28.99	67.35	30.53

Agar used, 2 per cent in distilled water.

Diameter of agar near electrode, 10 cm.

Diameter of agar at center, 2 cm. (approximately).

Diameter of electrode, 7.5 cm.

Distance between electrodes, 16 cm.

Duration of passage of current, 10 minutes.

Strength of current, 400 milliamperes.

only 2 cm. in diameter. Passage of a current of 400 milliamperes for 10 minutes from end to end of this cylinder caused the temperature of its center to increase by 45.78°C., while the rise in temperature at one end was 9.47°C., and at the other only 6.60°C. Table IV gives the temperature measurement in detail.

The experiment affords an admirable example of the effect of concentrating the lines of current flow on heat production. It is only by this maneuver that central heating can be obtained in a homogeneous medium in which heat loss occurs at a relatively uniform rate.

Many modifications of these two experiments were performed. In all of them maximal heating occurred at the point of greatest current concentration.

The Question of Skin Effect.

The term "skin effect" has no anatomical connotation. It is used by physicists to denote that property of a metallic conductor which causes nearly the whole of a high frequency current to pass along its surface rather than through its center. D'Arsonval (2) is said to have shown that this phenomenon is not encountered in electrolytes. Whether or not "skin effect" is encountered in biological conductors seems to be a moot point. We have previously reviewed the work of Dowse and Iredell (3), who found on the basis of both experimental and theoretical reasoning that "no measurable skin effect will be present" in the passage of a diathermy current through the human body.

Bettman and Crohn believe that they have presented evidence in favor of the view that skin effect is a factor in keeping the current near the surface of the body. The basis of their assumption lies in experimental observations made on sausages and cadavers. A current of known milliamperage was passed through a large bologna sausage. The core of the sausage was then enucleated, leaving just a shell of skin and meat, and the change in milliamperage was noted. They found that a decrease in 67 per cent of sausage weight resulted in only a 22 per cent decrease in current, and concluded, therefore, that "the skin effect, while not as marked as in metal, is certainly considerable." In our opinion their experimental method is not free from criticism. The hot wire milliammeter is at best a crude instrument for measuring current strength, varying in accuracy with the temperature of its environment. It can be easily shown, by measuring the rate of heat production in salt solution, that the current flow as estimated by a milliammeter is steadily diminishing, whereas the rate of heat production remains practically constant. Furthermore, if the current is turned off for a time and then turned on again without altering the spark gap, there is an apparent increase in milliamperage. These facts indicate that the milliammeter gives a false measurement of the current flowing through the conductor. We have found that simply interrupting the current flow for a while and then starting it without otherwise modifying any of the conditions may result in as much as a 70 per cent increase in milliammeter reading.

This same criticism, we believe, is applicable to experiments of a

similar nature which Bettman and Crohn performed on cadavers. We quote from their work:

"Electrodes of equal size were placed parallel and opposite to one another on either side of the abdomen. The current was turned on and the milliammeter reading noted. Then all the abdominal viscera were removed through a midline incision, the incision was sutured, and an attempt was made to preserve the contour of the abdomen by tension on one of the sutures. The current was again turned on, the electrodes and settings of the machine having remain untouched. It was found that the number of milliamperes recorded by the machine was identical. This cadaver experiment was repeated several times with similar results. It appears, therefore, that the viscera play little or no rôle in the consumption and, therefore, in the conduction of the current."

Several other sources of error appear to us to exist in this experimental procedure. First, the change in resistance produced by removal of the viscera may not be of an order of magnitude measurable by the ammeter. Secondly, it is very difficult to preserve the contours of the abdomen after viscerectomy.

We have performed an experiment of a similar nature on the dog's cadaver, but one which we believe to be free from the sources of error just enumerated. As well as estimating current flow by means of a hot wire milliammeter, we measured subcutaneous heat production by means of thermocouples. Moreover, we chose the thorax, a bony structure of which permitted no change in the contour. We did not eviscerate the thorax, but simply collapsed the lungs by introducing air into the pleural cavities through a hollow needle. This was done without interrupting the current flow or indeed without touching the diathermy machine. The results of two such experiments are in agreement. In each, five thermocouples were placed at various points in the subcutaneous or intramuscular tissue of the chest wall, while a diathermy current was allowed to pass between electrodes applied to the sides of the shaved thorax. Nine of the ten thermocouples showed a greater rate of heating after artificial pneumothorax than before. This indicates that when the lungs were collapsed more current passed through the superficial layers of the body. Moreover, in both experiments the collapse of the lungs was simultaneously accompanied by a drop in milliammeter reading. We reproduce in Table V the temperature readings of the thermocouples in both experiments, together with the average milli-

ammeter readings before and after pneumothorax. Fig. 3 presents graphically the change in the rate of heating recorded by one of the thermocouples and the accompanying change in milliammeter readings which occurred after the lungs were collapsed. This particular thermocouple lay in the insertion of the pectoral muscles on the anterior aspect of the midsternum.

On the basis of the average thermocouple readings published in the table it can be estimated, by the application of Joule's law, how much more current passed through the superficial tissues after collapsing

TABLE V.

Changes in Temperature and Milliammeter Reading after Artificial Pneumothorax in Experiments 5 and 6.

Position of thermocouple	Temperature increment during 15 min. of current flow			
	Experiment 5		Experiment 6	
	Before pneumo- thorax	After pneumo- thorax	Before pneumo- thorax	After pneumo- thorax
	°C.	°C.	°C.	°C.
On anterior surface of sternum at center.....	2.58	10.90	2.76	9.33
On left edge of sternum.....	7.43	10.74	7.85	7.65
On right edge of sternum.....	3.48	10.71	4.30	8.23
In deep muscles of back, 1 inch to right of midline.....	1.62	3.66	1.19	2.52
In deep muscles of back, 1 inch to left of midline.....	1.06	2.84	2.77	4.32
Average.....	3.23	7.77	3.77	6.41
Average reading on milliammeter.....	1250	1114	1233	1094

the lungs than before. In Experiment 5 this amounted to an increase of 35.7 per cent; in Experiment 6, to 24.2 per cent. These figures must represent the minimum percentage of current which was passing through the lungs. The values are probably too low, since in both experiments the milliammeter showed approximately an 11 per cent reduction of total current delivered to the cadaver after pneumothorax, and some current must have been passing through the collapsed lungs. We may conclude, therefore, that in the dead dog, at least, the diathermy current does actually pass through the interior of the body. The absence of true "skin effect," however, does not necessitate

the passage of all of the current through the deep tissues. As we have stated above, the current will take the path of least electrical resistance. Maximal heating occurs where there is greatest ohmic resistance, least heat loss, and above all, greatest concentration of the current. Where this will occur in the living animal body can be discovered only by experiment. The next part of the paper will deal with this aspect of the problem.

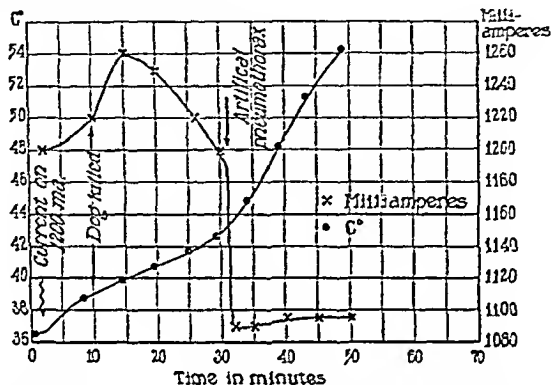


FIG. 3. Curve showing fluctuations of subcutaneous temperature and milliammeter reading before and after artificial pneumothorax.

Ordinates (left) = °C.

" (right) = milliamperes.

Abscissæ = time in minutes.

Part II. Experiments on the Living Body.

A dog's lung can be heated by diathermy only slightly (about 0.4°C.) above the rectal temperature (4). This is sufficient to cause the blood passing through the pulmonary capillaries to be warmed, with the result that the blood in the left ventricle, normally cooler than the blood in the right auricle, becomes, in fact, during diathermy, slightly warmer (5). These considerations might be taken as evidence for the penetration of the current through the deep structures of the body. Such a conclusion is, however, unwarranted until it has been shown that the heating of the lung is not the result of conduction from the heated skin and chest wall. That lung heating can occur even when the thoracic wall is simultaneously cooled will be shown in this paper.

The experiments about to be reported are of three general types:

1. Those in which the heat gradient of the body was measured from without inward before and during diathermy.

2. Those in which the heat gradient of the body was measured from without inward before and during the application of local heat to the chest wall. A comparison was made with the heat gradient during diathermy.

3. Those in which lung temperature was measured during diathermy with simultaneous cooling of the chest wall.

TABLE VI.

Temperature Gradient in Anesthetized Dogs.

Experiment No.	Rectal temperature	Lung temperature	Intramuscular temperature	Subcutaneous temperature	Skin temperature under electrode
	°C.	°C.	°C.	°C.	°C.
D 13	33.50	33.24	33.10	32.77	31.86
D 27	35.21	35.17		33.60	
			36.99		
D 29	37.15	36.94	36.42		
			34.53		
D 30	35.14	35.05	34.93	34.08	33.83
D 31	35.31	35.21	35.21	34.63	34.48
D 32	36.21	36.13		34.96	34.17
D 33	35.51	35.39	35.64	33.91	32.98
D 34	36.57	36.39	35.47	35.97	34.61
Average.....	35.57	35.44	35.14	34.27	33.65

All these experiments were done for the same purpose, namely, to learn whether the diathermy current passes through the interior of the body or through its more superficial tissues only. It is our belief that the evidence furnished by the experiments justifies the conclusion that the current penetrates the body wall and passes through the interior.

I. The Temperature Gradient in the Body before and during Diathermy.

By means of thermocouples such as have been described we were able to measure the temperature gradient which exists in the body of the anesthetized dog. Under normal conditions the rectal temperature is the highest, the lung, intercostal muscles, subcutaneous tissue,

and skin following in order. Table VI shows the temperatures recorded at various depths in eight different dogs. The averages were: Rectal 35.57°C ., lung 35.44°C ., intramuscular 35.14°C ., subcutaneous tissue 34.27°C ., and skin 33.65°C .

When cooling of the dogs occurred the heat loss progressed, as might be expected, more rapidly in the superficial layers than in the interior of the body.

With the passage of the diathermy current between electrodes placed laterally against the chest wall this temperature gradient promptly reversed, the hottest regions now lying nearest to the electrodes. The skin immediately under the electrode, of course, had the opportunity of losing heat by radiation, and perhaps offered a lower resistance to the current as well, and therefore did not heat up as fast as the subcutaneous tissue. This, however, and the thoracic musculature, showed a far greater elevation in temperature than either the lungs or the rectum. It was not uncommon to see a sudden jump in the subcutaneous temperature of over 10°C . in the first 5 minutes of diathermy with a simultaneous rise in rectal and lung temperature of only 0.3° to 0.5°C . (Experiment D 27).

The reversal in temperature gradient is admirably shown in Experiment D 13, Fig. 4.

The curves show the normal relationship at the start of the observations, the highest temperature being in the rectum, followed in turn by lung, intercostal muscle, subcutaneous tissue, and skin. During the preliminary period before the current flow was begun the animal lost heat, and the cooling, as will be seen in the figure, was more rapid in the superficial than in the deep parts. A current of 1200 milliamperes was then turned on. Immediately the skin and subcutaneous temperatures rose precipitously, the latter now exceeding the former. Intercostal muscle temperature now occupied a position between lung and superficial temperature, that of the lung in turn exceeding the rectal. It will be understood, of course, that in all these experiments the elevation in rectal temperature is due to heat conveyed to the rectum by the blood.

Later in the experiment the dog's skin was cooled by wetting his coat and blowing a stream of air on it. There resulted a drop in all temperatures, both internal and external. With the cessation of the cooling the superficial temperatures began again to mount, but the internal temperatures still continued to fall, since the blood which had been cooled on the surface was still circulating through the deep vascular tissues. On suddenly killing the animal the lung and intramuscular temperatures shot up steeply, since these structures, well insulated against heat loss by radiation, had been robbed of their normal medium for cooling, namely, the circulating blood.

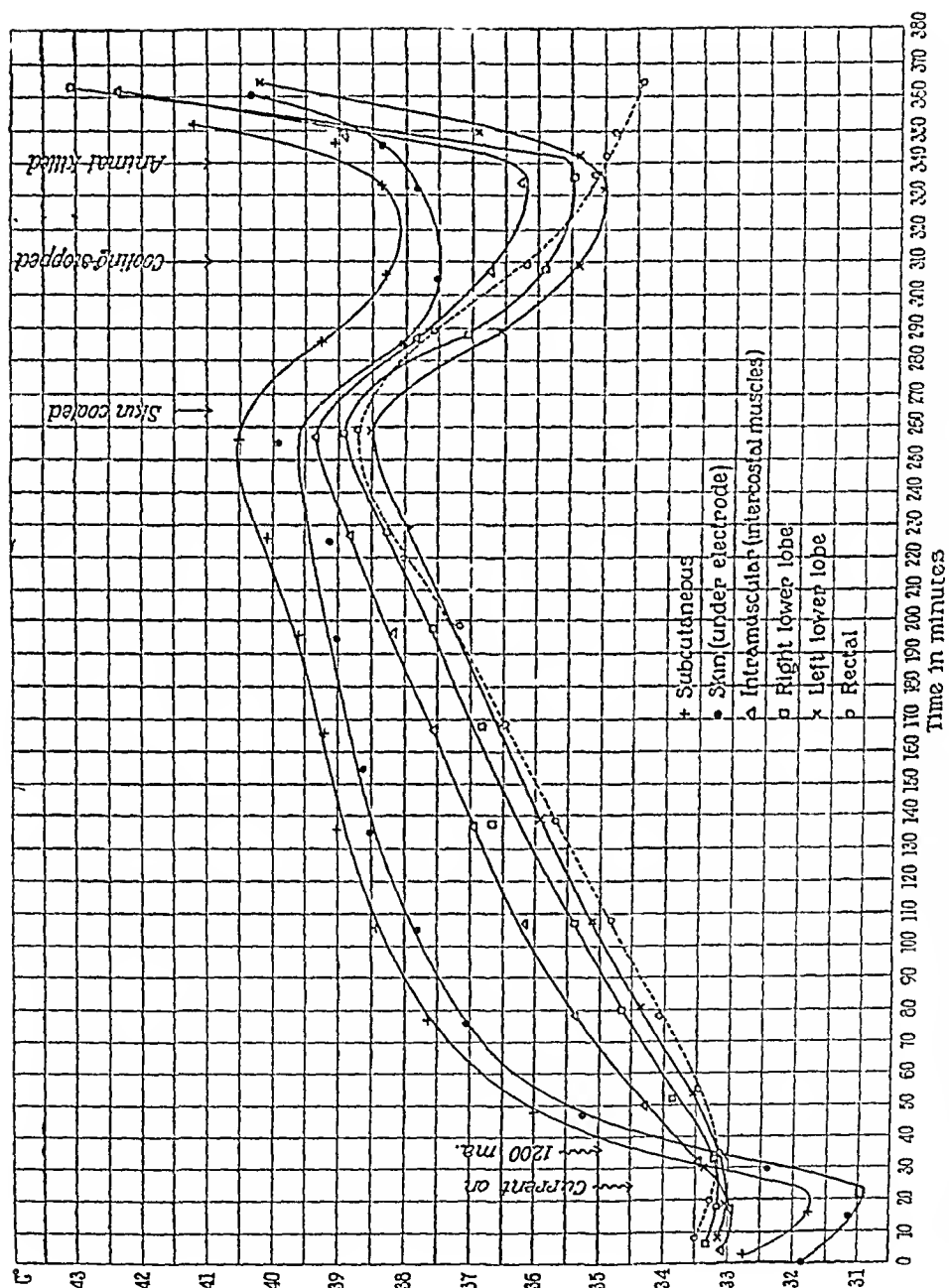


FIG. 4. Curve showing reversal of heat gradient after onset of diathermy and effect of cooling and death on superficial and deep temperatures.

Ordinates = °C.

Abscissæ = time in minutes.

This experiment, and many others of a similar kind, shows that *the normal heat gradient of the body is reversed during diathermy. Heating by diathermy occurs from without inward.* To find out whether deep heating is wholly the result of conduction from the heated chest wall a comparison was made between the effect of the application of local heat to the skin and the effect of diathermy on deep temperatures.

II. A Comparison of Deep Temperatures, during Application of Local Heat to the Skin, with Deep Temperatures during Diathermy.

For the application of local heat to the skin we prepared hollow reservoirs of brass, 3 X 4 inches in area, and slightly curved to fit the contours of the dog's chest wall. Through these reservoirs water was irrigated at the desired temperature. By soldering copper wires to the reservoirs they could be connected with the leads of the diathermy machine, and thus converted into electrodes.

With this equipment and the use of thermocouples it was easy to compare the deep temperatures produced by the local application of heat with the deep temperatures produced by diathermy. The results of such experiments showed that even though the skin temperature was higher with local heat than with diathermy, the subcutaneous and deep temperature elevation was less.

A specific example of this is shown in Experiment D 33. Water electrodes were fastened to an anesthetized dog's thorax. The temperature of the water circulating through the electrodes was maintained between 50° and 54°C. After an hour and 20 minutes exposure to local heat of this grade the recorded temperature changes were: skin under the electrodes, 32.52° to 45.23°C.; subcutaneous tissue, 33.43° to 41.28°C.; left lung, 35.16° to 35.04°C.; right lung, 35.11° to 35.01°C.; rectal temperature, 35.23° to 35.00°C. The failure of lung and rectal temperatures to rise under these circumstances should be noted.

The hilum of the left lung was then suddenly tied by fastening a previously placed ligature so that the circulation through this lung was obliterated (6). This was followed by a rise in temperature of the left lung of 0.20°C. and a gradual further rise during the next hour of 0.56°C. During this period the temperature of the right lung did not change. The flow of hot water was then discontinued and a diathermy current of 1200 milliamperes immediately started, which resulted in a 3°C. fall in skin temperature and, in spite of this, a 2°C. rise in subcutaneous temperature. The temperature of the normal lung, unchanged for more than an hour, now rose by 0.2°C., while the ligated lung temperature suddenly jumped

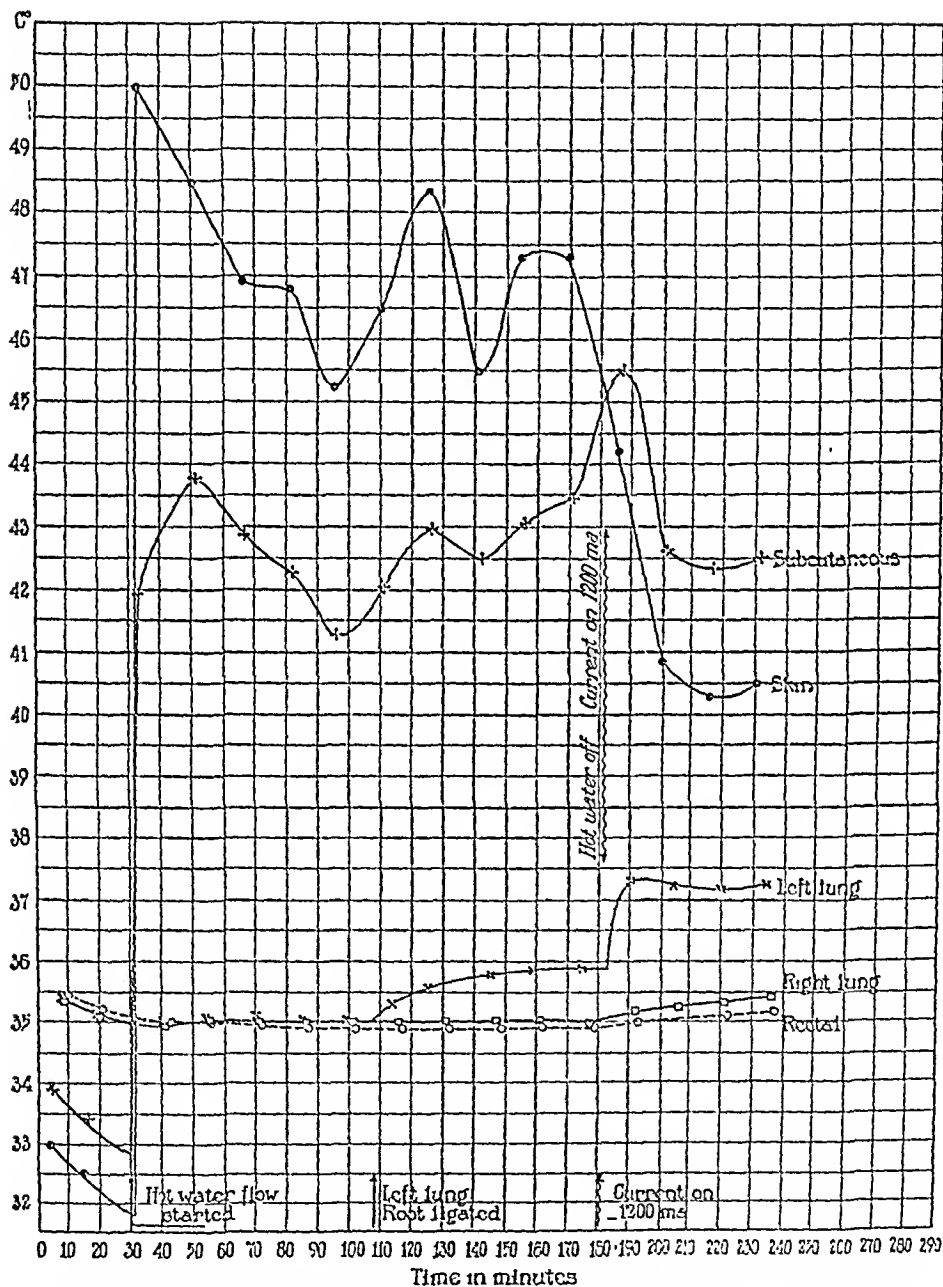


FIG. 5. Curve showing comparison of the effect of local heat with the effect of diathermy on superficial and deep temperatures.

Ordinates = °C.

Abscissæ = time in minutes.

from 35.86° to 37.29°C. The temperature changes in this experiment are graphically shown in Fig. 5. In addition to the changes just described the figure shows the characteristic upward inclination of rectal and normal lung temperature with the onset of diathermy.

This experiment may be regarded as evidence in favor of the belief that the deep heating of diathermy is due not only to local heating of the chest wall but to actual passage of the current through the deep tissues.

The final proof for this statement is, we believe, supplied by the next experiment.

III. Measurement of Lung and Rectal Temperature during Diathermy with Simultaneous Cooling of the Chest Wall.

Experiment D 42.—A female collie, weighing 8.75 kilos, was anesthetized by the intravenous injection of barbitol sodium (0.31 gm. per kilo). The thorax was then shaved and lead-tin electrodes measuring 3 × 4 inches were applied laterally. Great care was taken to avoid any edge effect by having the electrode surfaces as nearly parallel as the contours of the chest wall would permit. Six thermocouple needles were then inserted into the subcutaneous and intramuscular tissue of the chest wall so that they lay in the zone between the electrodes encircling the chest wall. This permitted temperature measurement in the whole circumference of the chest wall. A seventh thermocouple needle was thrust through the chest wall so that its point lay buried in the substance of the ventral lobe of

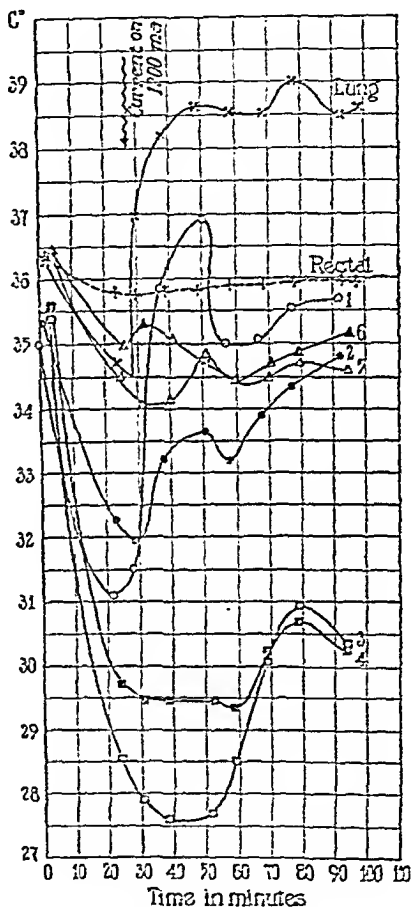


FIG. 6. Curve showing subcutaneous and intramuscular temperatures of the chest wall as well as rectal and lung temperatures during diathermy with simultaneous cooling of the chest wall.

The position of the thermocouples, 1 to 6, is shown in Table VII.

Ordinates = °C.

Abscissæ = time in minutes.

TABLE VII.
Temperature Changes Recorded in Experiment D 42.

Time	Position of thermocouples						Rectal thermometer	Current	Remarks
	1	2	3	4	5	6			
	Subcutaneous under center of right electrode	Under right pectoral muscle, beneath ventral edge of right electrode	In subcutaneous fat on mid-sternum	Under left pectoral muscle beneath ventral edge of left electrode	Against right ribs parallel to vertebral column under deep muscles of back	Buried in deep muscles of left back parallel to vertebral column	In substance of ventral lobe of right lung		
	°C.	°C.	°C.	°C.	°C.	°C.	°C.		
11:58-12:03	35.05	35.36	35.49	35.61	36.45	36.45	36.39		Ice caps in place
12:20-12:24	31.11	32.30	28.55	29.71	34.50	35.00	35.83		
12:25-12:30	31.51	31.98	27.91	29.47	34.07	35.31	35.75	On at 12:25 1200 ma.	
12:35-12:38	35.85	33.24	27.62	29.47	34.16	35.07	35.82		Ice cap to right side readjusted
12:45-12:49	36.94	33.64	27.70	29.47	34.85	34.77	35.85		
12:55-12:58	35.05	33.22	28.52	29.38	34.48	34.43	35.91		
1:05-1:09	35.09	33.90	30.07	30.25	34.50	34.73	35.92		
1:15-1:18½	35.57	34.35	30.96	30.72	34.73	34.88	35.97		
1:30-1:33	35.70	34.82	30.38	29.75	34.60	35.18	36.00		
1:35							35.96	Off	
1:36							38.64		
1:36½							37.87		

the right lung. The entire thorax was then surrounded by rubber ice caps, filled with a salt-ice mixture, held firmly against the chest wall by a binder. Rectal temperature was recorded by a mercury thermometer. After preliminary temperature measurements, a diathermy current of 1200 milliamperes was turned on and allowed to flow for an hour. Temperature readings on all thermocouples were made at 10 minute intervals.

The measurements show that both lung and rectal temperatures exceeded the temperatures developed in the chest wall. The fact that the rectal temperature was almost $3^{\circ}\text{C}.$ below lung temperature in this experiment can be accounted for by the cooling effect on the rectum of surface blood. This surface blood, counteracting the warming effect of the deep blood, explains the relatively stable rectal temperature, which ordinarily rises during diathermy. The thermocouple positions and readings are shown in Table VII, and in Fig. 6 there is a graphic representation of this experiment.

There can be no doubt that deep heating has occurred in spite of surface cooling; from which it must be concluded that the current passes through the interior of the body.

SUMMARY AND CONCLUSIONS.

The principles governing the passage of high frequency currents through various conductors have been discussed and exemplified in experiments done on both non-living and living bodies.

In Part I it was shown: (1) That the current takes the path of least electrical resistance rather than the shortest path; (2) that maximal heating occurs at the point of greatest concentration of the lines of current flow. In a homogeneous medium with parallel electrodes maximal heat production occurs in those portions of the medium adjoining the electrodes and the heat gradient is from without inward. Under these circumstances maximal heating never occurs at the center. In discussing the localization of heat not only the electrical resistance and current concentration, but also the cooling effect, must be considered.

In experiments on the dog's cadaver no evidence of the so called "skin effect" could be demonstrated. This is in contradistinction to the findings of Bettman and Crohn, but the discrepancy is explained on the basis of what we believe to be a technical error in their work.

The finding of no "skin effect" is in agreement with the conclusions of Dowse and Iredell, based on both experimental and theoretical considerations.

In Part II three types of experiments were performed on the anesthetized dog. The conclusions to be derived from them are these: (1) The heat gradient of the body is reversed during diathermy and heating occurs from without inward; (2) deep heating during diathermy is greater than that which results from the application of local heat to the skin; (3) the lung can be heated by diathermy in spite of simultaneous cooling of the chest wall.

These experiments we regard as satisfactory evidence of the passage of the current through the interior of the body.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

VIII. THE ANTIGENIC PROPERTIES OF HEMOLYTICALLY ACTIVE AND HEMOLYTICALLY INACTIVE MODIFICATIONS OF PNEUMOCOCCUS HEMOTOXIN.

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INTRODUCTION.

The present paper reports a comparison of the antigenic (antibody-invoking and antibody-combining) properties of the hemolytically active (reduced) form of pneumococcus hemotoxin with the antigenic properties of the inactive modifications derived from the original antigen (hemotoxin) by oxidation and by heat. The following inactive modifications were studied: (1) the reversible inactive oxidation product which can be reconverted to the active substance by test-tube reduction; (2) the irreversible, inactive products formed by treatment with strong oxidizing agents (exposure to strong concentrations of H_2O_2 and to sunlight); (3) the irreversible inactive products formed by heat.

The bacterial hemotoxins, as a class, are of immunological interest since they belong to the same large group of antigens ("antitoxinogens") as the important true toxins. Previous papers (1-4) in this series established certain relations between the hemolytic activity and the oxidation-reduction state of several bacterial hemotoxins. The present study of pneumococcus hemotoxin deals with the effect of oxidation and of heat upon the following properties which are possessed by the "true" toxins as well as by hemotoxins: (1) the cell-injuring property of the hemotoxin (including the cell combination reaction which precedes the injury), (2) its antibody-invoking property *in vivo*, and (3) its antibody-combining

* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

property *in vitro*. The results are reported in two papers; the present paper reporting the study of the antigenic properties, and the following paper reporting the study of the hemolytic or "cell injury" property.

EXPERIMENTAL.

General Methods.

Source of the Hemotoxin.—Pneumococcus extract (prepared by previously described methods (5)) supplied the hemotoxin used in the experiments. Enrichment of the broth culture medium by the addition of Avery's yeast extract (6) resulted in a particularly strong preparation, not only in hemotoxin concentration but also in oxidizing and reducing properties.

Titration of Antihemotoxin in Immune Serum.—The antihemotoxin potency was determined by adding different amounts of serum to a constant dose of hemotoxin; the hemotoxin-serum mixtures were shaken, and then incubated for 45 minutes at 25°C. to allow time for combination of hemotoxin and its neutralizing antibody; a constant amount of blood cells was then added and the final test systems incubated at 38°C. for 1 hour to determine the presence of free or unneutralized hemotoxin. 5 "units" (five times the amount of extract required to hemolyze completely 2.5 cc. of a 1 per cent suspension of washed rabbit cells) were used as the constant "dose" of hemotoxin instead of the 3 units employed in a previous study (7). This has the advantage of obscuring the inhibitory effect of normal serum, which is due to non-specific lipoid constituents unrelated to the true and specific neutralizing antibody.

The previously described (7) controls were included to prove that none of the apparent neutralization was due to oxidative inactivation during the incubation of the hemotoxin-serum mixtures previous to the introduction of the blood cells. It is also important that there was no visible protein precipitation in our hemotoxin-serum mixtures, since this phenomenon, if it had occurred, might have introduced errors by the mechanical removal of active hemotoxin.

Hemotoxin Inhibition by Normal Serum.—Although it is well known that normal serum contains constituents that inhibit the usual bacterial hemotoxins, it is desirable to emphasize the fact that these substances are not related at all to the true immune neutralizing antibody. The immune pneumococcus antihemotoxin is species-specific and does not inhibit the hemotoxins of tetanus or of the Welch bacillus (7) while the normal serum constituents are non-specific, and inhibit to some extent almost all the bacterial hemotoxins.

In our experiments, the normal serum of each animal, obtained before immunization, was included as a control in the antihemotoxin titration of the immune serum. The small amount of immune serum required for hemotoxin neutralization is of an entirely different order of magnitude than the relatively large amount required for the comparable inhibition of hemolysis by normal serum. Hence, while a certain part of the apparent neutralizing capacity of

immune serum is always due to normal constituents of serum, there is little chance for confusion between the non-specific inhibition and the specific neutralization by immune serum, if the dose of hemotoxin and the amount of serum are properly chosen.

Toxicity of the Extracts Employed in the Immunization.—The pneumococcus solution used to supply the hemotoxin contained, in addition to the hemotoxin and other pneumococcus constituents, the endocellular toxic products described by Cole (8). While we believe that these toxic substances are distinct from the hemotoxin, it was necessary to consider them a possible source of complication in the immunization of the animals.

The results of tests of the toxicity of the extract employed in this study showed that intravenous injections must be limited to relatively small doses, while amounts as large as 0.4 cc., if desired, could be injected by the subcutaneous route without injury to rabbits.

Preliminary Experiments on the Influence of Size of Dose of Antigen (Hemotoxin) upon the Antibody (Antihemotoxin) Response.

Comparisons of the effectiveness of different modifications of any antigen should consider the factor of the size of dose employed in the immunization. This becomes especially important in comparisons of the effectiveness of toxoid solutions containing traces of the active substance with the effectiveness of solutions of the active substance itself, for if there were no relation at all between dosage and response, the traces of active substance in the toxoid solution might be responsible for the antibody production. Hence, it was considered an essential step in the present investigation to determine whether or not there was a direct relationship between the dosage of antigen (hemotoxin) and the antibody (anti-hemotoxin) response; and if such a relation existed, in what zone of antigen dosage it held valid. It was assumed that the quantitative relation, if it existed at all, could be demonstrated best by keeping the amount of antigen well below that required for the maximum immunity response, for if the amounts of antigen were so large that even the smallest test doses were sufficient to invoke the maximum response, any quantitative relation would be obscured.

Since it was sufficient for the objects of the subsequent major experiments to show immunity differences resulting from large differences in magnitude of the antigen dosage, the following experiments were limited to two sizes of dose of antigen, one of which was ten times as great as the other.

Four male rabbits of approximately the same age and weight were selected. The test doses of antigen were chosen to be 0.2 cc. and 0.02 cc. of the hemotoxin solution given subcutaneously in order to keep even the larger amount somewhat below that required for the maximum immunity response. Two rabbits were immunized with the 0.2 cc. dose and two others with the 0.02 cc. dose. Injections were made daily for 6 successive days with a test bleeding 8 days later. A second

course of injections, begun immediately after the first bleeding, consisted of five injections on successive days with a test bleeding after a rest period of 9 days. The results are given in Table I.

The results of this experiment (Table I) show that the amount of antigen (hemotoxin) injected does affect the antibody (antihemotoxin) response, provided the dose of antigen is kept within proper limits (that is, when the larger doses are not excessive, and when the smaller doses are near the minimum required for the stimulation of a detect-

TABLE I.

Influence of Size of Dose of Antigen (Hemotoxin) upon Antibody (Antihemotoxin) Response.

Immunization		Response (antihemotoxin titrations of serum)							
Rabbit	Size of dose; amount of bacterial extract in each injection	Hemolysis by 5 "units" of hemotoxin which had been incubated in presence of serum for 45 min. at 25°C., before addition of red blood cells							
		Serum obtained before immunization	Serum obtained after first course of injections		Serum obtained after second course of injections				
		0.05 cc.	0.05 cc.	0.013 cc.	0.05 cc.	0.013 cc.	0.006 cc.	0.003 cc.	0.002 cc.
	cc.								
1	0.2	++++	0	++	0	0	0	0	+
2	0.2	++++	0	++	0	0	0	±	++
3	0.02	++++	++++	++++	0	++	++++	++++	++++
4	0.02	++++	++++	++++	0	+++	++++	++++	++++

0 = no hemolysis.

± = trace of hemolysis.

+

++ = hemolysis approximately one-half complete.

+++ = hemolysis approximately three-fourths complete.

++++ = complete hemolysis.

able response). In view of the well known differences in the immunity response of different individual animals, one cannot expect too much, but the relationship between dosage and response was sufficiently definite to be utilized in the following major experiments which compare the antigenic properties of different modifications of pneumococcus hemotoxin.

Comparison of the Antibody-Invoking Properties of the Reduced (Active) and the Reversible Oxidized (Inactive) Forms of Pneumococcus Hemotoxin.

This experiment consisted of a comparison of the antibody-invoking properties of the hemolytically active (reduced) hemotoxin and of its inactive, reversible oxidation products. In the "unoxidized" solution, the hemotoxin was preserved

TABLE II.

Proportion of the Hemolytically Active (Reduced) and Hemolytically Inactive (Reversibly Oxidized) Modifications of the Hemotoxin Contained in the Solutions Employed in the Immunizations.

Amount of hemotoxin solution	Unoxidized hemotoxin solution		Oxidized hemotoxin solution	
	Measurement of active hemotoxin; hemolytic activity of solution before treatment with reducing agent	Measurement of active hemotoxin plus the reversibly oxidized form; hemolytic activity of solution after treatment with reducing agent	Measurement of active hemotoxin; hemolytic activity of solution before treatment with reducing agent	Measurement of active hemotoxin plus the reversibly oxidized form; hemolytic activity of solution after treatment with reducing agent
cc.				
0.03	++++	++++	+++	++++
0.02	++++	++++	±	++++
0.01	++++	++++	0	++++
0.005	++++	++++	0	++++
0.001	+++	+++	0	++++
0.0005	+	+	0	+
0.0002	0	0	0	0

0 = no hemolysis.

± = trace of hemolysis.

+

++ = hemolysis approximately one-half complete.

+++ = hemolysis approximately three-fourths complete.

++++ = complete hemolysis.

in its original active or reduced form by the reducing properties of the system in the absence of air. In the "oxidized" pneumococcus extract (prepared by exposing the extract to air for about 24 hours at 37°C.), the reducing properties of the system had been destroyed and the oxidizing agents (peroxides) had converted the hemotoxin to hemolytically inactive oxidation products; these inactive oxidation products were reversible, and could be reconverted to the original active form of the hemotoxin by biological or chemical reducing agents.

All the material employed in the immunization was analyzed in order to deter-

mine what proportion of the total amount of antigen was in the active (reduced) state and what proportion was present in the inactive form; this was particularly important in the case of the "oxidized" or "inactive" material which (like most toxoid material) did contain residual traces of the active hemotoxin. The dosage employed in the immunization was kept low in order to stay within the zone in which there is a direct relation between the amount of antigen and the degree of immunity response. These two conditions were essential: (1) knowledge of the nature of the material used in immunization, (2) control of antigen dosage, since the crux of the experiment consisted not only in a comparison of the antibody response to the equal amounts of the unoxidized and of the oxidized solutions, but also in determining whether or not the residual traces of active hemotoxin persisting in the oxidized solution were in themselves sufficient to invoke a significant response.

1. *Analysis of the Material Utilized in the Immunization.*—One series of animals was immunized with "unoxidized" hemotoxin solution, and another series with the solution which had been oxidized by exposure to air for 24 hours at 37°C. The two solutions were examined by methods (1-4) which measure both the reduced and reversibly oxidized forms of the hemotoxin.

The results of these tests (Table II) show that in the unoxidized solution, all the hemotoxin was in the reduced or active form. In contrast to the unoxidized solution, almost all the hemotoxin in the oxidized solution was present in the reversibly oxidized form, as indicated by the great increase in the hemolytic activity of this solution when treated with the reducing agent. It is important to note that residual traces of the active (reduced) form did persist in the "oxidized" solution; the amount of active substance, however, was small, since 0.02 cc. of the oxidized extract gave less hemolysis than did 0.0005 cc. of the unoxidized material.

The total antigen (hemotoxin) in the two extracts was the same, since their hemolytic activity was approximately identical when measured after the reduction treatment. The unoxidized extract can be assumed to have contained 100 per cent of the total antigen in the active or reduced state. In the oxidized solution, it is sufficient for the present purpose to estimate that more than 90 per cent of the total antigen was present in the oxidized (inactive) state, and that the traces of the reduced (active) form represented less than 10 per cent of the total hemotoxin. (This estimate of 10 per cent is purposely generous; 0.03 cc. of the oxidized solution possessed no more hemolytic activity than 0.001 cc. of the same solution after reduction treatment.)

2. *Control of Dosage in the Immunization.*—Eight rabbits, all obtained from the same breeder, and all of approximately the same weight, were selected; four were immunized with the unoxidized and four with the oxidized material.

Two doses were employed in the injection of the animals of each series; the larger dose (0.2 cc.) of the unoxidized and oxidized solutions for the respective series was somewhat less than that required for the maximum response, and the smaller dose (0.02 cc.) was near the minimum required for any detectable response.

These two doses, chosen from the results of previous experiments (Table I), presented a convenient basis for a valid evaluation of the antibody-invoking properties of the oxidized (inactive) and reduced (active) forms of the hemotoxin, the quantitative relations between them being so arranged that the traces of reduced (active) hemotoxin contained in the larger test dose of the oxidized material were quantitatively less than the amount of the reduced substance contained in the smaller test dose of the unoxidized material.

The routine of the immunization was the same as previously described; the antibody response was determined by measurements of the antihemotoxin content of the immune sera after one and two courses of injections. Since the quantitative relations between dosage and antibody response were more valid in the sera obtained after the first course of injections than in those obtained after the second course, the objects of the experiment are satisfied by presenting in Table III a summary of the results after the first course of the immunization.

TABLE III.

Comparison of the Antibody-Invoking Properties of the Reduced (Hemolytically Active) and of the Reversibly Oxidized (Hemolytically Inactive) Forms of Pneumococcus Hemotoxin.

Condition of the antigen (hemotoxin) used in the immunization	Immunity response (5 "units" of hemotoxin tested against 0.05 cc. of the immune serum obtained after 1 course of injections)	
	Immunization with 0.02 cc. doses	Immunization with 0.2 cc. doses
	Neutralization	Neutralization
100 per cent of the total antigen in reduced (hemolytically active) state	0	+
Over 90 per cent of the total antigen in the reversibly oxidized (hemolytically inactive) state; less than 10 per cent of antigen in the reduced (active) state	0	+

Table III presents two important facts. First, one course of injections of 0.2 cc. of either the unoxidized or oxidized material invoked the production of significant amounts of antihemotoxin; second, the same number of injections of 0.02 cc. (one-tenth the size of the first dose), proved insufficient to cause a significant immunity response. In spite of the fact that 100 per cent of the total antigen (hemotoxin) was in the reduced state in the one solution and 90 per cent was in the oxidized state in the other solution, like immunity responses were invoked in all instances by equal doses of the unoxidized and oxidized

material. The lack of any essential difference in the degree of response to the larger or effective dose (0.2 cc.) of the two solutions containing such widely different proportions of the two modifications of the antigen, indicates that there is no essential difference in the antibody-invoking properties of the reduced and of the reversibly oxidized forms of the hemotoxin. Since it was known that less than one-tenth of the total hemotoxin (reduced plus oxidized forms) was in the reduced state in the oxidized solution, the fact that the same number of injections of 0.02 cc. of the unoxidized solution (or one-tenth the larger dose of the solution containing 100 per cent active hemotoxin) did not invoke antibody production, serves to prove that the residual traces of reduced substance remaining in the oxidized materials were by themselves insufficient to account for the antigenic effectiveness of the larger dose (0.2 cc.) of the oxidized hemotoxin solution. Although the possibility has not been eliminated that the effectiveness of the oxidized form may be increased by the presence of traces of the reduced form, the above evidence indicates that the antibody-invoking properties of the reduced and reversibly oxidized forms of the antigen are identical; and that the loss of the hemolytic property is not necessarily accompanied by any loss in the capacity to invoke antibody production.

Experiments on the Antibody-Invoking Property of Inactive, Irreversible Modifications of the Antigen (Hemotoxin) Derived by Means of Strong Oxidizing Agents.

The preceding experiment showed that the loss of hemolytic activity by the reversibly oxidized modification of the hemotoxin was not accompanied by any detectable change in its antigenic effectiveness. The reversibly oxidized form is the product obtained when the hemotoxin is oxidized by the peroxides produced when this type (9) of bacterial solution is exposed to air. However, other modifications of the hemotoxin are formed when the bacterial solutions are treated with strong oxidizing agents; these products agree with the reversibly oxidized form in that they have lost the original hemolytic property, but differ in that they cannot be reconverted to the original form by test-tube reduction. The irreversible, inactive modifications are not so easily produced from pneumococcus hemotoxin as are the similar products derived from the hemotoxins of tetanus and Welch bacilli (2, 3). (With the latter substances, the irreversible products are formed so readily that it is difficult to induce the formation of the reversible product without at the same time causing the formation of some of the irreversible products.)

Experiments were made to determine whether the changes in pneumococcus hemotoxin which are involved in the formation of the irreversible products cause a loss of the antibody-invoking property. The hemotoxin solution was exposed to 0.5 molar H_2O_2 ("Dioxogen" diluted in phosphate solution) for 7 days at 37°C . It was desired to eliminate the H_2O_2 at the end of this period of exposure so that it would not interfere with the reduction tests to be made for the presence of the reversible product of the hemotoxin. In order to accelerate the "spontaneous" deterioration of the H_2O_2 , the mixture was exposed for 3 days to intense summer sunlight in addition to the 7 days storage at 37°C . in the dark. After this treatment, all the hemotoxin had been converted to irreversible modifications and the H_2O_2 destroyed.

Four rabbits were immunized with the doses used in the preceding experiment, but none of them developed any detectable antihemotoxin.

The results showed that the irreversible products formed by treatment with strong H_2O_2 were devoid of the antibody-invoking property of the original hemotoxin. While there is a striking contrast between the antigenicity of the reversibly oxidized modification and the non-antigenicity of the irreversible products studied in this particular experiment, it is possible that other irreversible products which retain antigenicity are formed by other agents than those employed in our experiments. It must be remembered that drastic treatment was employed to convert the first formed reversible modification to the irreversible modification. Treatment of any antigenic substance with high concentrations of H_2O_2 is likely to cause profound changes in the molecule,¹ changes indeed which frequently involve hydrolysis or splitting of the molecule as a whole in distinction to oxidation of individual groupings without profound changes in other parts of the molecule. Hemoglobin, for example, when treated with the proper oxidizing agent, is converted quantitatively to methemoglobin (also a reversible product), but if treated with a high concentration of H_2O_2 , a part of the hemoglobin (or more properly, a part of the methemoglobin) is split to globin and hematin (10). One must distinguish

¹ While the exposure to 0.5 molar H_2O_2 which results in loss of antigenicity of the hemotoxin would also partially convert hemoglobin to (reputedly) antigenically ineffective products, it was not an unreasonably drastic treatment with which to treat the hemotoxin. It will be shown in a later paper that the same treatment does not result in a comparable loss of the antigenicity of certain other protein substances which give rise to anti-pneumococcus-protein precipitins.

material. The lack of any essential difference in the degree of response to the larger or effective dose (0.2 cc.) of the two solutions containing such widely different proportions of the two modifications of the antigen, indicates that there is no essential difference in the antibody-invoking properties of the reduced and of the reversibly oxidized forms of the hemotoxin. Since it was known that less than one-tenth of the total hemotoxin (reduced plus oxidized forms) was in the reduced state in the oxidized solution, the fact that the same number of injections of 0.02 cc. of the unoxidized solution (or one-tenth the larger dose of the solution containing 100 per cent active hemotoxin) did not invoke antibody production, serves to prove that the residual traces of reduced substance remaining in the oxidized materials were by themselves insufficient to account for the antigenic effectiveness of the larger dose (0.2 cc.) of the oxidized hemotoxin solution. Although the possibility has not been eliminated that the effectiveness of the oxidized form may be increased by the presence of traces of the reduced form, the above evidence indicates that the antibody-invoking properties of the reduced and reversibly oxidized forms of the antigen are identical; and that the loss of the hemolytic property is not necessarily accompanied by any loss in the capacity to invoke antibody production.

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between these two types of changes in the treatment of antigens with different oxidizing agents. Pneumococcus hemotoxin, for example, is converted to a hemolytically inactive but antigenically effective modification when the oxidizing agents are properly chosen, as shown by the above results, but treatment with other oxidizing agents yields a product which is not only hemolytically inactive, but also antigenically ineffective.

It is important not to emphasize any apparent correlation between the reversibility of the hemolytic property of hemotoxoids and their retention of antigenicity, for while the two products differ from each other from the standpoint of hemolytic activity, in that one is reversibly inactive and the other is irreversibly inactive, this is probably by no means the only difference between them. Indeed, from a fundamental point of view, the reversibility of the one product and the irreversibility of the second are important simply as an index that the first product has suffered less profound molecular changes, and hence is more likely to retain the antigenic properties of the original substance. The dangers entailed in the assignment of antigenic effects to prominent toxic properties are evident from analogy with the antigenic properties of hemoglobin, methemoglobin, and globin (11, 12).

Although methemoglobin has lost the most prominent property of the original hemoglobin, the loss of the oxygen-combining property caused by the change in the valency of the iron is not accompanied by changes in the antigenically effective groups of the complex protein molecule. Since the oxygen-combining property is just as important a property to hemoglobin as is hemolysis to the reduced hemotoxin, it is no more remarkable for the reversibly oxidized hemotoxin to undergo changes affecting the hemolytic property without alteration of its antigenically active groupings. Similarly, the loss of antigenicity by the irreversible products of the hemotoxin is probably due not to the fact that it does not regain hemolytic activity upon reduction, but simply to the fact that the hemotoxin molecule as a whole has undergone profound changes which include alteration of the antigenically effective groupings; just as the reputed lack of antigenicity on the part of globin is due not to the fact that it cannot regain its oxygen-combining activity, but to the fact that it is a split product of the original antigenic hemoglobin.

Experiments on the Antibody-Invoking Properties of Heat-Inactivated Modifications of the Hemotoxin.

Two rabbits were immunized with hemotoxin solution which had been inactivated by exposure to 55°C. for 6 minutes and two others with solution boiled for 10 minutes. One animal in each series was given two courses of subcutaneous injections of 0.2 cc.; this was equivalent to the maximum immunization employed in previous experiments. The immunization of the other animal in each series was continued for 7 months, and consisted of eight courses of five or six injections of 0.2 cc. with one rest period of 2 months in addition to the usual periods of 8 or 9 days between each course.

None of the animals immunized with the heat-inactivated modifications developed any detectable antihemotoxin. The failure to respond to the prolonged immunization indicated that the loss of antigenicity was absolute. While a quantitative decrease in the effectiveness of heated antigens is common experience, the complete loss of antigenicity after short periods of exposure to 55°C. is an unusual example of the effect of heat upon bacterial substances.

Antibody-Combining Properties of the Different Modifications of Pneumococcus Hemotoxin.

That the reduced (hemolytically active) form of the antigen possesses the property of combining with the antibody, can be regarded as an established fact, since its neutralization by combination with the antihemotoxin was the basis of the tests of the antihemotoxin content of the immune serum studied in the preceding experiments. The object of the following experiments was to determine whether or not the different modifications of the hemotoxin (antigen) which have lost the hemolytic property of the original antigen, still retain the property of combining with the antibody.

The experiments consisted of a comparison of the amount of immune serum required to neutralize a constant dose of active hemotoxin under the following four sets of conditions: (1) test mixtures containing the constant dose of active hemotoxin but no inactive derivatives of the hemotoxin; (2) test mixtures containing an equivalent amount of the reversibly oxidized form of hemotoxin in addition to the dose of active hemotoxin; (3) test mixtures containing an equal amount of the irreversibly "oxidized" products in addition to the active hemotoxin; (4) test mixtures containing an equal amount of the heat-inactivated products in addition to the active hemotoxin.

Four solutions were prepared, 1 cc. of which contained, respectively: Solution

I: 5 "units" active hemotoxin; Solution II: 5 "units" of active hemotoxin plus 5 "units" of the reversibly oxidized form; Solution III: 5 "units" of active hemotoxin plus 5 "units" of the irreversibly oxidized products; Solution IV: 5 "units" of active hemotoxin plus 5 "units" of heat-inactivated products. 1 cc. amounts of the four solutions were put into different series of tubes. Dilutions of immune serum (containing antihemotoxin) were prepared so that 1.0 cc. of the respective dilutions contained the following increments of serum: 0.10, 0.067, 0.050, 0.040, 0.030, 0.020, 0.016, 0.010 cc. 1 cc. of each of the serum dilutions was then added to the four series of tubes which contained, respectively, Hemotoxin Solutions I, II, III, IV. The rest of the procedure was identical with that in previous experiments, the four series of hemotoxin-serum mixtures being incubated for 45 minutes at 25°C. before the addition of the red blood cells.

Controls of Active Hemotoxin Content of the Solutions Utilized in the Neutralization Tests.—It was essential to prove that the different hemotoxin solutions did not differ significantly in their content of active hemotoxin solution when tested in the absence of immune serum. This control was especially important in the case of Hemotoxin Solution II since there frequently are significant traces of the active or reduced form persisting in the oxidized extracts containing the reversibly oxidized form of the hemotoxin. (The oxidized extract itself was examined for the presence of the active or reduced hemotoxin, and was chosen for use in this experiment because of the almost complete absence of active lysin, 0.20 cc. of the undiluted extract causing only traces of hemolysis in 2.0 cc. of 1 per cent red blood cells; the amount (0.015 cc.) used in the experiment proper was entirely without hemolytic effect.)

The results are condensed in Table IV.

Several experiments of another type were also made in which the active hemotoxin was added to previously incubated mixtures of serum and inactive hemotoxin, instead of incubating the immune serum with mixtures of active and inactive hemotoxin. From analogy with the Danysz phenomenon which occurs in the fractional addition of toxin in toxin-antitoxin mixtures, one would expect this second set of conditions to emphasize the difference between the amount of serum required to neutralize the active hemotoxin alone and the amount required for the active hemotoxin plus inactive forms which unite with the antihemotoxin. Our results in the latter type of experiments, however, were not significantly different from those obtained when the active hemotoxin was added to the serum mixture at the same time as the hemotoxoid. Since the results were practically the same as those shown in Table IV, no protocol need be presented.

Table IV shows that when the reversibly oxidized products were present in the serum mixture during the period allowed for neutralization of the active hemotoxin, a larger amount of immune serum was required for the neutralization than when an equal dose of the active form of the hemotoxin was incubated with serum alone; but that the

presence of either one of the other two hemolytically inactive modifications was without effect upon the neutralizing dose of the immune serum. These results indicate that the reversible oxidation products

TABLE IV.

Antibody-Combining Properties of Different Modifications of Pneumococcus Hemotoxin; Effect of the Presence of Inactive Modifications of the Hemotoxin upon the Amount of Immune Serum Required for the Neutralization of a Constant Dose of the Active Hemotoxin.

Hemotoxin solutions	Tests of removal of effective antibody (antihemotoxin) by combination with inactive forms of the antigen (hemotoxin)	Controls; to prove that all test solutions contained approximately the same amount of the hemolytically active form of the hemotoxin					
		Hemolysis by constant dose of the hemotoxin solution when incubated in presence of different amounts of immune serum, before addition of red blood cells			Hemolysis by different amounts of the hemotoxin solutions, tested in absence of immune serum, against 2.5 cc. of 1 per cent red blood cells		
		Amount of serum, cc.			Amount of hemotoxin solution, cc.		
		0.067	0.030	0.016	0.24	0.12	0.08
Solution I	5 units of active hemotoxin alone	0	0	+++	+++	+	0
Solution II	5 units of active hemotoxin plus an equivalent amount of its inactive, reversibly oxidized products	+	++++	++++	+++	+	0
Solution III	5 units of active hemotoxin plus an equivalent amount of its inactive, irreversibly oxidized products	0	0	+++	+++	+	0
Solution IV	5 units of active hemotoxin plus an equivalent amount of its heat-inactivated products	0	0	+++	+++	+	0

0 = no hemolysis.

± = trace of hemolysis.

+ = hemolysis approximately one-fourth complete.

++ = hemolysis approximately one-half complete.

+++ = hemolysis approximately three-fourths complete.

++++ = complete hemolysis.

of the hemotoxin, although they have lost the cell injury (hemolysis) property, retain the property of uniting with the antibody; and that the other "inactive" modifications of the hemotoxin have lost not only the hemolytic property but also the property of combining with the antibody.

It is significant that the same two modifications of the hemotoxin, the hemolytically active reduced form and the hemolytically inactive, reversibly oxidized form, which possess the property of invoking antibodies *in vivo*, are also the ones which combine with the antibody *in vitro*; and that the other two modifications, which are devoid of the antibody-invoking property, are likewise devoid of the antibody-combining property. The same correlation between the two properties of antigens seems to be established for diphtheria toxin since it is the basis of the Ramon flocculation method for measurement of the total antigenically effective products (toxin plus effective toxoid).

It is important to note that the "constant dose" of active hemotoxin in the four hemotoxin solutions in Table IV is based upon measurements which detect only the active form of the hemotoxin. Thus, while all the solutions contain the same amount of the hemolytically active form, the amount of total hemotoxin (*i.e.*, active plus inactive modifications) is in fact only half as great in Hemotoxin Solution I (where all the "total hemotoxin" is active) as in Hemotoxin Solutions II, III, IV (where only half of the "total hemotoxin" is hemolytically active). With the true toxins, when the toxin content is based upon M.L.D., the conditions are much the same (*i.e.*, the M.L.D. detect only the part of the "total toxin" which is still in the "active" form); and the amount of immune serum required for the neutralization of a "constant dose" of the "active" antigen is likewise increased by the presence of its non-toxic but antigenically effective modifications.

DISCUSSION.

The following modifications of the antigen (hemotoxin) were studied: (1) the original, active substance; (2) the inactive, reversible oxidation product formed by exposure of hemotoxin solutions to air; (3) the inactive, irreversible products formed by prolonged treatment with high concentrations of H_2O_2 ; (4) the inactive products formed by heat.

The first experiments compared the antibody-invoking property of the hemolytically active (reduced) form of the antigen with that of the hemolytically inactive, reversible oxidation products. Two series of

animals were immunized; one series, with unoxidized hemotoxin solutions in which 100 per cent of the total antigen was present in the reduced state; a second series, with oxidized solutions in which more than 90 per cent of the total antigen was present in the reversibly oxidized state. Equal doses of the oxidized and unoxidized solutions invoked like immunity responses, and the traces of the reduced hemotoxin contained in the larger doses of the oxidized solution were shown to be quantitatively insufficient to account for the antibody production. Although it is possible that the antigenic effectiveness of the oxidized form was increased by the presence of traces of the reduced form, the evidence indicated that the antibody-invoking properties of the reduced and reversibly oxidized forms of pneumococcus hemotoxin are identical, and that the hemolytic property of the antigen is not essential to the stimulation of the neutralizing antibody.

In contrast to the apparent identity of the antibody-invoking properties of the reduced and reversibly oxidized forms of the hemotoxin, the irreversible products formed by treatment with strong H_2O_2 proved devoid of antigenic properties; but the failure of antibody response to the type of strongly oxidized material used in these experiments does not constitute evidence that the reversibility of the inactive products is correlated with the antigenic function, for other irreversible products possessing antigenic properties might be formed by other agents. The important fact is that the products yielded by one oxidation treatment (that induced by the peroxides formed in the aerated bacterial extract) were hemolytically inactive but antigenically effective, and that the products yielded by more drastic treatment were devoid of antigenic properties. Thus, unless the treatment be properly chosen, the antibody-invoking properties may be destroyed by the processes employed to destroy the toxic properties of the original antigen. This fact is of interest in connection with the mechanism of toxoid immunization. In many instances when toxoids are used for immunization, the response is most effective when traces of the original active substance are present in the material injected into the animals; and it is sometimes suggested that these traces of active substance, although by themselves quantitatively insufficient to invoke antibody production, may accelerate the response to the non-toxic toxoid. However, from analogy with the differences in anti-

genicity of the different modifications of pneumococcus hemotoxin, it is probable that the less effectiveness of toxoid fluids which contain no traces of active toxin is due simply to the fact that a considerable portion of the antigenically effective toxoids are always transformed to ineffective products whenever the treatment is violent enough to inactivate all traces of active toxin.

The results of the comparison of the different modifications of hemotoxin from the standpoint of the antibody-combining property were similar to the results of the comparison of their antibody-invoking properties. The same modifications of the hemotoxin (the reduced and the reversibly oxidized forms) which possessed the antibody-invoking property also possessed the antibody-combining property; and the other two modifications which were devoid of antibody-invoking properties *in vivo* were likewise devoid of the antibody-combining property *in vitro*. The fact that one of the modifications which possessed the two antigenic properties was hemolytically inactive is evidence that the groups of the hemotoxin molecule in which the antibody-combining property and the antibody-invoking property are resident are not necessarily altered by processes which inactivate the molecular groupings responsible for the cell injury function of the original antigen. The lack of antigenic properties on the part of the other two hemolytically inactive modifications is evidence that the treatment employed to alter the toxic property of the molecule must be properly chosen to avoid profound changes in the molecule which affect antigenically effective groupings. In this sense, from a purely immunological aspect, the reversibility of the hemolytic activity of the antigenically effective oxidation product of pneumococcus hemotoxin is important, simply as an index that the change of the antigen molecule has not been a profound one.

The fact that some of the non-toxic modifications of antitoxinogens can retain the antigenic properties of the original toxic antigen raises the question of whether or not there is any essential *immunological* difference between the antitoxinogen or primarily toxic type of antigen and the sensitizing or non-toxic type of antigen.² The most

² Zinsser's (13) separation of the antitoxinogens from other antigens serves its intended purpose as a convenient basis for the inclusive treatment of the sensitizing antigens in a single group, but it should not be misinterpreted to

fundamental distinction between them lies in the fact that the antitoxinogens alone have the property of combining with and injuring specific cells, which is a pharmacological rather than an immunological property. All antigens must possess the fundamental property of stimulating the animal body to produce a specific antibody, and the method of stimulation may be the same for all.

indicate essential differences between the two groups of antigens from the standpoint of antibody stimulation, nature or mode of antigen-antibody reaction, or other immunological reactions. The distinction is based almost entirely upon the specific cell-injuring properties of the one group and the absence of such properties in the other—a criterion not intended to separate the antitoxinogens from other antigens regardless of the possibility of more fundamental chemical and immunological relationships.

The conception of the "essential identity" of sensitizing antibodies, derived from one antigen but detected in different systems, can in many respects be projected to indicate fundamental likenesses between all antibodies. The usual distinction between the two types of antibodies (antitoxins and sensitizing antibodies) depends upon differences between the properties of the antigen-antibody compounds and the properties of the specific antigens. With the toxin-antitoxin compounds, the *prominent* change is in pharmacological properties; with the other antigen-antibody compounds, it is usually a change in physical properties. But the physical properties of toxin-antitoxin can also differ from those of the toxin as exemplified in the Ramon flocculation; and some hypersensitive phenomena might be interpreted as due to the acquisition by the sensitizing antigen-antibody compounds of pharmacological properties not possessed by the antigens. In a certain sense, "neutralization" as well as "agglutination" can be considered as a phenomenon secondary to the actual combination of antigen and antibody. The lack of toxicity of toxin-antitoxin compounds is essentially a fortunate circumstance employed to detect the combination of these antigens with their antibodies, just as the change in physical properties is a convenient means of observing the union of agglutinin with agglutinin. If differences in the test systems frequently obscure the identity of the sensitizing antibodies in spite of the essential likeness of their antigen-antibody combinations, differences in the test systems may also obscure fundamental likenesses between the antitoxinogens and other antigens.

While the quantitative relations between antitoxinogens and antitoxins are usually more distinct than with other antigens, the application of the rule of "neutralization by multiple proportions" to the explanation of many phases of toxin-antitoxin titration curves or to the Danysz phenomenon is just as difficult as the explanation of the quantitative relationships between the other antigens and their antibodies.

Although no definite evidence is available, it is highly doubtful, at least in the case of some antitoxinogens, whether the particular cells for which they are specifically toxic have anything to do with antibody production. Hemotoxins, for example, are considered to belong in the antitoxinogen class of antigens because they have the specific property of combining with and injuring erythrocytes, but from their nature it is unlikely that red blood cells can be the agents of antibody production. If the mechanism of antibody production with antitoxinogens is essentially the same as with sensitizing antigens (*i.e.*, a similar response to stimulation by specific antigenic groupings of a foreign protein rather than a defensive response to stimulation by the groups responsible for the toxic property), then the antigenicity of modifications of toxic antigens would be determined by the same conditions that determine the antigenicity of derivatives of all antigens. While the specific toxicity is the most prominent property of all antitoxinogens, the loss of one prominent and characteristic property without loss of antigenicity is a set of conditions which also occurs among the sensitizing antigens. For example, the oxygen-combining property is just as important and prominent a property of the hemoglobin molecule as is the specific cell injury property of toxin molecules; and yet, the loss of the oxygen-combining property by methemoglobin is not accompanied by loss of the antigenic properties of hemoglobin. While it cannot be said that the pharmacological action of toxins has no more to do with the production of antitoxin than the oxygen-combining property of hemoglobin has to do with the production of antihemoglobin precipitins, the prominence of any one particular property of a complex molecule implies no relation to its antigenicity. Fundamentally, the antigenicity of toxoids requires no other explanation than that for methemoglobin, *i.e.*, that any substance can lose any one of its properties without loss of antigenicity provided the alteration does not include changes in the antigenically effective groupings nor render it insoluble in the body. From this standpoint, the antigenic effectiveness of the reversibly oxidized modification of the hemotoxin requires no assumption of the possibility of its reconversion to the hemolytically active form by *in vivo* reduction.

On the other hand, one cannot dismiss the possibility that the cells of certain tissues might be able to reconvert the inactive derivatives

to the original active substance. While evidence is lacking that the reversibility of toxoids is related at all to antigenicity, the different cells and different tissues of the body present a variety of systems which are known to differ in acidity and other factors, and which consequently might differ in their capacity to reconvert inactive toxoids to the original form of the antigen. It does not seem at all impossible for at least some of the cells or tissues responsible for antibody production to present the requisite conditions for the reversal of toxoids to the original toxin, and yet for the particular cells with which the toxin is pharmacologically reactive to be devoid of the conditions required for the reversion. However, conjectures from this aspect are futile in view of the almost complete lack of knowledge of the site of antibody production.

SUMMARY.

The following modifications of the antigen (pneumococcus hemotoxin) were studied: (1) the hemolytically active (reduced) substance; (2) the hemolytically inactive, reversible oxidation product; (3) the inactive irreversible products formed by treatment with high concentrations of H_2O_2 ; (4) the inactive products formed by heat. The antibody-invoking property of the reversibly oxidized form seemed to be identical with that of the original, hemolytically active or reduced form; neither of the other two hemolytically inactive products invoked antibody production. The same modifications of the antigen which exhibited the antibody-invoking property *in vivo* possessed the antibody-combining property *in vitro*; and the modifications which lacked the one property also lacked the other. Evidence is presented that the groups of the hemotoxin molecule in which the true antigenic properties are resident are not necessarily altered by processes which inactivate the groupings responsible for the toxic (hemolytic) action of the original antigen. The lack of antigenic properties on the part of the other two hemolytically inactive modifications is evidence that the treatment employed to alter the toxic property of the molecule must be properly chosen to avoid profound changes which affect the antigenically effective groupings. From an immunological point of view, the reversibility of the antigenically effective oxidation product of pneumococcus hemotoxin is important as an index that the loss of

toxicity (hemolysis) was accomplished without a profound change in the molecule.

The theoretical significance of the antigenicity of non-toxic modifications of toxic antigens is discussed.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

IX. THE ERYTHROCYTE-COMBINING PROPERTY OF PNEUMOCOCCUS HEMOTOXIN.

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INTRODUCTION.

The preceding paper (1) dealt with a comparison of different modifications of pneumococcus hemotoxin, from the standpoint of properties (antibody-invoking and antibody-combining) which are possessed in common by all true antigens. The present paper deals with the cell injury (hemolysis) property of the hemotoxin, a property which in a sense is the criterion of distinction between the antitoxinogens and other antigenic substances, the specific toxicity of the hemotoxin for erythrocytes being analogous to the selective toxicity of diphtheria and tetanus toxin for other tissue cells.

Hemolysis by pneumococcus hemotoxin, like cell injury by true toxins, includes two reactions: (1) combination of the toxic substance with the cell for which it has specific affinity; (2) injury of the cell with which it has combined. The following investigation dealt especially with the combination of the hemotoxin with the erythrocyte. Experiments were made to determine whether the reversibly oxidized form, although hemolytically inactive, retains the property of combining with red blood cells. Since it was possible to separate the combination and injury reactions, a number of experiments were also made upon the factors involved in the erythrocyte-combining reaction of the active form of the hemotoxin. Although these experiments were not suitable for a study of the kinetics of the hemolytic reaction, a number of salient facts were revealed which are of some theoretical

interest in connection with the mechanism of hemolysis by pneumococcus hemotoxin.

EXPERIMENTAL.

Absorption of Oxidized and Unoxidized Hemotoxin Solutions with Red Blood Cells.

It is known that the reduced form of the hemotoxin is hemolytically active and that the reversibly oxidized form is hemolytically inactive (2). The following experiment was made to determine whether the reversibly oxidized modification can combine with the blood cell even though it cannot induce its final hemolysis. In a previous paper (3) the combination or union of the active form of the hemotoxin with the blood cell was demonstrated by experiments similar to those employed by earlier workers with other hemotoxins. The new feature introduced in the following experiment consists in the application of reduction treatment to the absorbed fluids as a means of determining whether or not the hemolytically inactive oxidized hemotoxin had been removed from the test system by combination with the blood cells. This method offered a means of obtaining more direct evidence of the combining properties of the hemolytically inactive hemotoxoids than has hitherto been possible.

Two solutions of hemotoxin were used in the experiments: (1) an oxidized solution in which almost all the hemotoxin had been converted to the reversibly oxidized (hemolytically inactive) modification; (2) an unoxidized solution which contained only the reduced or hemolytically active form of the hemotoxin. Each of these solutions was diluted with 19 volumes of sterile salt solution. The dilution was necessary for two reasons: to prevent hemolysis during the absorption period since hemolysis occurs even at 0°C. if the hemotoxin concentration is too great; and to retard the oxidation of the reduced or active hemotoxin since the rate of oxidation is directly related to the concentration of the bacterial extract. One half of each solution (oxidized and unoxidized) was absorbed with erythrocytes; the other half was not absorbed and served as a control.

The procedure in the absorption proper was essentially the same as in the previous study (3). 3 cc. of blood cells were added to 30 cc. of the diluted oxidized solution and likewise to the diluted unoxidized solution. In order to test the effect of absorption at different time intervals, 7 cc. samples were removed from each of the tests and controls at the end of 1, 2, and 3 hours, and centrifuged to remove the blood cells. The hemotoxin titrations of the supernatant fluids included measurements of the fluids before and after treatment with the reducing agent employed to convert the hemolytically inactive oxidized form to the original active substance.

Since the separation of the "combination" and "injury" reactions is entirely dependent upon differences in the speeds of the two reactions at low temperatures, the most rigid temperature precautions are essential to the success of experiments

of this type. In addition to holding the absorption mixtures themselves in an ice bath at 0°C., all apparatus (pipettes, etc.) was kept in the ice box at 2°C.; the entire manipulation including the centrifugation was also carried out in a room at this temperature.

The absorption of the reduced (active) hemotoxin from the unoxidized solution was complete in all the tests, and the protocol of the experiment (Table I) includes only the results of the absorption of the oxidized hemotoxin solution.

TABLE I.

Effect of Absorption with Red Blood Cells upon the Hemotoxin Contained in Oxidized Hemotoxin Solutions.

Amount of hemotoxin solution	Hemotoxin measurements before treatment with reducing agent		Hemotoxin measurements after treatment with reducing agent				
			Unabsorbed control (1 hr. and 3 hrs.)	Solutions receiving one absorption treatment			"Reabsorbed" solution
	Unabsorbed control (1 hr. and 3 hrs.)	Absorbed with erythrocytes (1 hr. and 3 hrs.)		Absorbed for 1 hr.	Absorbed for 2 hrs.	Absorbed for 3 hrs.	
cc.							Supernatant fluid of mixture absorbed for 3 hrs., reabsorbed with fresh cells for a second period of 3 hrs.
0.12	0	0	++++	++++	++++	++++	++++
0.06	0	0	++++	+++	+++	+++	+++
0.04	0	0	++++	++	++	++	++
0.02	0	0	++++	+	+	+	+
0.01	0	0	++++	0	0	0	0
0.002	0	0	+	0	0	0	0

0 = no hemolysis.

+ = hemolysis approximately one-fourth complete.

++ = hemolysis approximately one-half complete.

+++ = hemolysis approximately three-fourths complete.

++++ = complete hemolysis.

The results showed a distinct difference in the effect of absorption upon the hemotoxin contained in the oxidized and in the unoxidized solutions. The active, reduced hemotoxin was completely removed from the unoxidized solution at the end of 1 hour's absorption with red blood cells at 0°C., while as shown in Table I, the absorption treatment removed only part of the hemotoxin in the oxidized solutions.

One important point to be decided is whether or not the removal of

hemotoxin from the oxidized solution depends upon an intrinsic ability of certain oxidized modifications of the hemotoxin to combine with the blood cell without causing its hemolysis. The proportion of the total hemotoxin removed by the absorption is too large to be explained by the direct combination of the slight traces of active hemotoxin which probably always persist in reversibly oxidized solutions at the end-point of the oxidative reaction. It might still be suggested that the hemotoxin removed from the oxidized solution consists of reduced hemotoxin obtained by a gradual reversal of the oxidized form during the absorption period. However, this explanation does not seem tenable for, if it were true, the time of absorption should influence the degree of completeness with which the hemotoxin was removed; and the actual results (Table I) show that the hemotoxin was removed as completely at the end of 1 hour as at the end of 3 hours. Moreover, the blood cells themselves when resuspended after their removal from the oxidized hemotoxin absorption mixtures do not hemolyze as would be the case if it were the reduced or active form of hemotoxin which had combined with the erythrocytes.¹

The above facts indicated that the hemotoxin removal was due to a real combining property of the oxidized hemotoxin. The next

¹ In contrast to the prompt reactivation of the hemolytic property when solutions of the oxidized form are treated with a reducing agent, negative results were always obtained in attempts to reactivate the oxidized hemotoxin after it had combined with the erythrocytes. Although a number of such experiments were made, the conclusiveness of the results may be complicated to some extent by the fact that the O_2 contained in the HbO_2 of the blood cells made it necessary to employ relatively large amounts of $Na_2S_2O_4$ in order to establish reducing conditions in the test systems.

Further experiments are necessary in order to settle this question, but the fact that it is possible to reconvert the oxidized hemotoxin to the active form by reduction when in solutions by itself, does not indicate at all that the oxidized hemotoxin can likewise be reduced after it has combined with the blood cell. It is known that free (deoxygenated) hemoglobin is much more readily oxidized than is hemoglobin after it has combined with either oxygen or carbon monoxide (4, 5). We have also obtained evidence (6) that the reduced hemotoxin is oxidized with greater difficulty when combined with the blood cell than when it is in solution alone; and it is equally possible that the oxidized hemotoxin when combined with the erythrocyte is in a condition which renders its reduction more difficult.

question to be decided was why only part and not all, of the oxidized form was removed by the absorption treatment. Two possibilities were suggested: (1) that the final equilibrium between the free oxidized hemotoxin and that combined with the blood cell is different from that obtaining for the reduced hemotoxin, and that a considerable amount of the oxidized hemotoxin remains uncombined when the equilibrium is established whether at the end of either 1 or 3 hours; (2) that the reversibly oxidized hemotoxin exists in different modifications, some possessing and others lacking the property of combining with the blood cell. In order to determine which of these possible explanations was valid, an oxidized hemotoxin solution was subjected to two repeated absorptions, the supernatant fluid of the first absorption mixture being reabsorbed with fresh blood cells for a second period of 3 hours. The results of this experiment are summarized in the last column of Table I, and show that the oxidized hemotoxin left in the solution after the first absorption was not appreciably affected by the second absorption. These results indicated that the reversibly oxidized hemotoxin, which we formerly considered as one modification of the hemotoxin, consists in fact of at least two different modifications, both lacking the complete property of hemolysis, but only one retaining the property of combining with the erythrocyte.

In previous papers, the oxygen-combining activity of hemoglobin and the corresponding inactivity of methemoglobin have been compared to the hemolytic activity of reduced hemotoxins and the inactivity of their reversible oxidation products. It is interesting to observe that the existence of the two different modifications of the reversibly oxidized form of the hemotoxin makes it impossible to continue the analogy upon the basis of oxygen-combining and erythrocyte-combining properties. The reduced (ferrous) hemoglobin and the reduced hemotoxin both possess the combining property, and methemoglobin and the second modification of the reversibly oxidized hemotoxin both lack the combining property. But there is no known reversibly oxidized modification of hemoglobin which retains the original oxygen-combining property to correspond with the reversibly oxidized hemotoxin modification which retains the erythrocyte-combining property.

Absorption of a Constant Dose of Hemotoxin by Different Amounts of Red Blood Cells.

In connection with the investigation of the combining properties of the hemotoxin, a number of experiments were made upon the relation of the combining

reaction to the hemolytic action as a whole. The following experiment dealt with the effect of varying concentrations of blood cells upon the combination of the hemotoxin with the erythrocytes. Three tubes containing an equal amount of diluted hemotoxin solution were absorbed, respectively, with 0.1 cc., 0.5 cc., and 2.0 cc. of red blood cells. The constant amount of hemotoxin was known to be sufficiently great to cause the complete hemolysis of the largest amount of blood cells employed in the experiment, if incubated at 37°C. The crux of the experiment, therefore, consisted in determining whether or not smaller numbers of blood cells ($1/4$ and $1/20$ the maximum number) can combine with much larger amounts of hemotoxin than are required for their hemolysis.

10 cc. of diluted hemotoxin solution were added to each of four tubes: 0.1 cc. of blood cells was added to Tube 1; 0.5 cc. of blood cells to Tube 2; 2.0 cc. of blood cells to Tube 3; Tube 4 served as unabsorbed control. The absorption mixtures were held at 0°C., with frequent gentle stirring or shaking. Tests for the completeness of absorption were made at the end of 45 minutes, 6 hours, and 20 hours. The absorbed cells obtained from centrifuged samples were resuspended in cold (0°C.) salt solution. (All the supernatant fluid had been removed with capillary pipettes from the layer of sedimented cells to insure that no uncombined hemotoxin would be taken up in the resuspension of the blood cells. As an additional precaution, the walls of the tube and the surface layer of the cell residue were also washed carefully with cold salt solution.) The suspensions from the absorption mixtures where 0.5 cc. and 2.0 cc. of erythrocytes had been employed, were diluted with salt solution, so that all three suspensions had approximately equal concentrations of blood cells during the following tests for hemolysis.

The suspensions of absorbed cells (erythrocytes combined with hemotoxin) were then held for 30 minutes at 0°C., 30 minutes at 25°C., and 30 minutes at 38°C. The lower temperature of incubation was employed to minimize the possibility of inactivation of the hemotoxin during the lytic process. At the end of this incubation, the tests were centrifuged and the supernatants compared colorimetrically with hemoglobin standards prepared by complete laking of like suspensions of erythrocytes; the centrifuge tubes were also examined for the presence of unhemolyzed cells.

Although the above tests were made at the end of 45 minutes, 6 hours, and 20 hours, the absorption proved to be complete at the end of 45 minutes and the results of the three tests were the same.

Two facts were shown by the results of this experiment: (1) a constant dose of hemotoxin was as completely removed by combination with 0.1 cc., and 0.5 cc. of blood cells, as by absorption or combination with 2.0 cc. of blood cells; (2) this same amount of hemotoxin when combined with the blood cells was sufficient to set free all the hemoglobin from 20 times the minimum number of erythrocytes required

to combine with all the hemotoxin. These facts indicate that more than one hemotoxin molecule may combine with one red blood cell. Apparently, in mixtures containing equal concentrations of hemotoxin and different concentrations of erythrocytes, the hemotoxin molecules are distributed more or less uniformly among the available blood cells; if many blood cells are present in a unit volume, only a few hemotoxin molecules combine with one erythrocyte; if few blood cells are present, a large number of hemotoxin molecules combine with each of the available cells.

The experiment also showed that while practically all the hemoglobin was liberated from the largest number of erythrocytes as well as the smallest number, the amount of stroma which remained after the hemolytic process was dependent upon the concentration of blood cells used in the absorption, or in other words, upon the amount of hemotoxin combined with the individual erythrocytes. Apparently, the amount of pneumococcus hemotoxin required to liberate all the hemoglobin is much less than that required to cause a significant solution of the stroma. Rabbit erythrocytes seem to lose practically all their hemoglobin after even slight injury of the cell wall; the shadow or ghost cells obtained from mixtures where 95 per cent of the hemoglobin had been set free retained their original cell outline and showed no evidence of gross injury associated with the loss of hemoglobin.

It is obvious from these facts, that in two different hemolytic test systems, there may be obtained differences in the degree of solution of the stroma although the liberation of the hemoglobin is practically complete in both instances. Under these conditions, complete hemolysis becomes a question of whether or not stroma solution is included in the definition of hemolysis. In the present paper, the terms "hemolysis" and "hemolyze" when applied to the "hemolyzing" capacity of the hemotoxin refer to the liberation of hemoglobin without regard to differences in degree of solution of the stroma.

Influence of the Hemotoxin-Blood Cell Ratio upon the Rate of Hemolysis.

The preceding experiment dealt with the influence of the blood cell concentration upon the combination reaction, the first of the two steps involved in hemolysis by pneumococcus hemotoxin. The following experiment dealt with the influence of erythrocyte concentration upon hemolysis proper. The preceding experiment had indicated that when different amounts of blood cells are quickly mixed with a constant dose of hemotoxin, the hemotoxin distributes itself more or less uniformly among the available cells so that the number of hemotoxin molecules combining with the individual erythrocytes tends to be inversely proportional to the blood

cell concentration. In the present experiment, this principle was utilized in order to determine the influence of the relative number of hemotoxin molecules previously combined with the individual cells upon the rate of hemolysis. Thus, while a constant dose of hemotoxin was absorbed with different amounts of blood cells, the experiment consisted essentially in a comparison of the rates of hemolysis of blood cells with different amounts of hemotoxin combined with the individual erythrocytes. The hemolysis process itself was carried out at a low temperature (0°C.) in order to show differences in the rates of hemolysis to better advantage than at 37°C. where even the largest concentration of blood cells would be completely hemolyzed within 1 hour.

Three tubes containing 10 cc. of diluted hemotoxin solution were prepared: 2.0 cc. of blood cells were added to the first tube and 0.5 cc. and 0.1 cc. of blood cells to the second and third tubes, respectively. A like series of the same concentrations of blood cells was prepared in salt solution without hemotoxin to serve as controls on spontaneous hemolysis.

TABLE II.

Influence of the Hemotoxin-Erythrocyte Ratio upon Hemolysis Proper.

Concentration of red blood cells in test mixtures	Degree of complete hemolysis after different periods of time at 0°C.; per cent of total hemoglobin set free	
	45 min.	6 hrs.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	60	90
5	3	42
20	0.2	5

The above tubes were held in an ice bath at 0°C. as in previous absorption experiments. Representative samples were removed at the end of 45 minutes, 6 hours, and 20 hours and the percentage of complete hemolysis determined colorimetrically. The tests upon the controls (without hemotoxin) proved that there was less than 0.5 per cent spontaneous hemolysis in any of the suspensions of different erythrocyte concentration at the end of 45 minutes and 6 hours. Significant hemolysis had occurred in all the controls at the end of 20 hours, and to avoid the complication of compensating for spontaneous hemolysis, the results of the 20 hour tests are omitted from the protocol (Table II).

It is evident in Table II that the rate of hemolysis was inversely related to the blood cell concentration. This relationship is in conformity with the deductions made from the results of the previous experiment; *i.e.*, that the hemotoxin distributes itself more or less

uniformly among the available blood cells, the number of hemotoxin molecules which combine with each erythrocyte being inversely related to the erythrocyte concentration. Apparently, the rate of actual hemolysis of the blood cells is dependent upon the number of hemotoxin molecules combined with the individual erythrocytes; when large numbers of hemotoxin molecules are combined, hemolysis is rapid; if small numbers are combined, hemolysis is slow. In this sense, if one separates the two reactions involved in hemolysis by pneumococcus hemotoxin, it may be said that the rate of the second reaction (hemolysis proper) is actually determined by conditions imposed during the preliminary or combination reaction.

It is interesting to observe that the differences in rates of hemolysis were so pronounced, that not only was the percentage of hemolysis greater in the mixture containing the fewest number of blood cells, but the absolute amount of hemoglobin set free during the 1st hour was greater than in the test where the hemotoxin was allowed to act upon 20 times that number of blood cells. This apparently greater hemoglobin-liberating "capacity" of the same amount of hemotoxin during an equal time (1 hour at 0°C.) is the more interesting in view of the fact that the dose of hemotoxin employed was more than sufficient to cause the complete hemolysis of the largest amount of blood cells. After longer periods of time, of course, the absolute amount of hemoglobin set free was greatest in the test containing the largest amount of blood cells.

These relationships are of incidental interest in connection with the technic of absorption of the hemotoxin. Due to the inverse relation between blood cell concentration and rate of hemolysis, a constant dose of hemotoxin during the first few hours at 0°C. causes less total hemolysis the larger the amount of blood cells with which it has combined. Consequently, the separation of the hemotoxin from the bacterial solution without hemolysis in the supernatant fluid is less difficult when large amounts of blood cells are used in the absorption.

Absorption of a Constant Dose of Hemotoxin with the Stroma Derived from Different Amounts of Blood Cells.

Since it seemed likely that the combining property of the blood cell was a function of the stroma, the following experiment was made to compare the amount of stroma required for the absorption or combination of a constant dose of hemotoxin with the amount of blood cells which could be hemolyzed by the same dose of hemotoxin.

When rabbit erythrocytes are added to distilled water, much of the stroma is left after the liberation of practically all the hemoglobin. The stroma suspension used in this experiment was prepared by adding 1 cc. of packed erythrocytes to

50 cc. of distilled H_2O ; the mixture was left in the ice box overnight and the dissolved hemoglobin removed by centrifugation; the residue was shaken up again in 50 cc. distilled H_2O to permit hemolysis of any intact cells that might have remained; this process was repeated three times, so that the stroma or cell residue was subjected four times to the action of 50 volumes of H_2O . Although the residue appeared faintly pink when large amounts of the centrifuged sediment were examined, no significant amounts of hemoglobin were contained. The washed stroma (cell residue) was finally resuspended in 10 cc. of salt solution, 1.0 cc. of the stock suspension being considered equivalent to the stroma derived from 0.1 cc. of erythrocytes. Different dilutions of this suspension were prepared to furnish the desired amount of stroma for the absorptions. The procedure for the absorption with the stroma was essentially the same as that employed in the previous absorption experiments with erythrocytes.

The results of these experiments showed that the absorbing or combining capacity of the stroma derived from distilled water hemolysis of rabbit erythrocytes is approximately equivalent to that of the intact red cells themselves. After an absorption period of 1 hour at $0^\circ C.$, the stroma derived from approximately 0.025 to 0.030 cc. of erythrocytes sufficed to combine with an amount of hemotoxin sufficient to cause the complete hemolysis of about 0.80 cc. of the same lot of red cells; the stroma from 0.020 cc. or less amounts failed to remove all the hemotoxin. This is about the same relation between absorbing capacity and hemolyzing capacity that was found in the preceding absorption experiments with the whole blood cells. The marked absorbing or combining property of the stroma must be considered as an important factor in determining the inhibition of the hemotoxin hemolysis.

The stroma which had combined with the hemotoxin was resuspended in salt solution and incubated to determine whether the combined stroma would be dissolved in a manner analogous to the hemolysis of the combined erythrocytes. No lysis or solution of the combined stroma could be detected by gross examination except in the tests where the amount of stroma used for the absorption had proved insufficient to remove or combine with the test dose of hemotoxin. These results are in conformity with the observations made in connection with the previous experiment and indicate that practically all the hemoglobin may be set free from rabbit erythrocytes by action of pneumococcus hemotoxin without marked solution of the

stroma; and that significant amounts of stroma always remain undissolved unless great excesses of the hemotoxin are present.

Influence of the Presence of Dissolved Hemoglobin and of Stroma upon the Two Steps (Combination and Hemolysis Proper) Involved in the Hemolytic Action of Pneumococcus Hemotoxin.

It is a recognized fact that enzymes are inhibited by the products of their action. Northrop (7) has shown, especially for protein-hydrolyzing enzymes, that the inhibition is due to the combination of the hydrolytic products with the active enzyme. It is to be expected that the presence of the products of hemolysis would also affect the hemolytic activity of the hemotoxin, but it was desired to determine whether the inhibitory effect upon hemolysis as a whole was due to the influence of these products upon the preliminary combination of the hemotoxin with the blood cells, or whether the inhibition was due to the effect of the presence of the products of hemolysis upon the liberation of hemoglobin from cells previously combined with hemotoxin. The principal products of hemolysis of rabbit cells by the hemotoxin are the dissolved hemoglobin and the stroma which remains after the hemoglobin is set free. The following experiment was designed to compare the influence of each of these different products (hemoglobin and stroma) upon the two steps (combination and hemolysis proper) involved in hemolysis by the hemotoxin.

Influence upon Combining Reaction.—The influence upon the combination reaction was tested by adding a constant number of red blood cells to three tubes containing respectively: (1) a constant dose of diluted hemotoxin solution alone; (2) a constant dose of diluted hemotoxin solution to which a known amount of dissolved hemoglobin had been added; (3) a constant dose of diluted hemotoxin solution to which a known amount of stroma had been added. A large excess of hemotoxin was present (ten times that required to liberate all the hemoglobin from the blood cells used in the test); the amount of dissolved hemoglobin and the amount of stroma were small (each being equivalent to only one-half the hemoglobin or stroma derived from the amount of blood cells used in the test; or 1/20 that derived from the maximum amount of blood cells that the test dose of hemotoxin could hemolyze). These quantitative relations were designed to make a severe test upon the inhibitory influence of the presence of the hemoglobin and stroma, and it is obvious that if their presence had only a slight effect upon the combination reaction, it would not be detected at all. The blood cells were given an opportunity to combine with the hemotoxin in the three test systems, and then the absorption mixtures were centrifuged. The supernatant fluids were removed, and the sedimented cells rinsed with cold salt solution to remove all traces of the added hemoglobin and the added stroma. The entire procedure up to this stage had been carried out at 0°C. and no hemolysis had occurred in the test systems. The rest of the procedure consisted in allowing the cells which had been "absorbed"

under different conditions to hemolyze under the same conditions. This was done by resuspending them in equal amounts of fresh salt solution and incubating the suspension at 25°C. for 1 hour and at 38°C. for another hour, after which the percentage of complete hemolysis was determined.

Influence upon Hemolysis Proper.—In the experiment upon the influence of the presence of dissolved hemoglobin and of stroma upon the hemolysis of cells previously combined with hemotoxin, the quantitative relations between all the reagents was the same as described. But the conditions of the tests were essentially exactly the opposite; the three samples of blood cells were allowed to combine with

TABLE III.

Influence of the Presence of Dissolved Hemoglobin and of Stroma upon the Two Steps (Combination and Hemolysis Proper) Involved in the Hemolytic Action of Pneumococcus Hemotoxin.

Influence upon combination		Influence upon hemolysis	
Hemolysis of combined cells when resuspended in salt solution		Hemolysis of cells previously combined in absence of hemolytic products	
Conditions during combination period	Per cent total hemoglobin liberated during hemolysis period	Conditions during hemolysis period	Per cent total hemoglobin liberated during hemolysis period
Cells added to hemotoxin solution alone	100	Combined cells resuspended in salt solution alone	100
Cells added to hemotoxin plus hemoglobin solution	95	Combined cells resuspended in salt solution plus hemoglobin solution	100
Cells added to hemotoxin solution plus suspension of stroma	8	Combined cells resuspended in salt solution plus suspension of stroma	100

the hemotoxin in the absence of the products of hemolysis; the combined cells were then resuspended, respectively, in the following three test systems: (1) salt solution alone; (2) salt solution containing the same amount of dissolved hemoglobin that was used in the other experiment; (3) salt solution containing the same amount of stroma that was used in the other experiment. The same period of time was allowed for hemolysis of these three test mixtures as that employed in the preceding part of the experiment.

It is obvious that our comparison of the action of the hemotoxin in the different test systems is based upon the final end-point of the hemolytic reaction which furnishes a less delicate method of comparison than if it were based upon differences

in rates of hemolysis. The objects of the experiment are satisfied by presenting a summary of the results in Table III.

The results of this experiment (Table III) show that the presence of the products of hemolysis had a much greater influence upon the combination of the hemotoxin with the blood cells than upon the subsequent liberation of hemoglobin from erythrocytes which had previously combined with the hemotoxin. It is important to emphasize again that the conditions of the experiment were such as to detect only the most marked effects of the presence of the products upon either of the two stages of the hemolytic reaction. The test dose of hemotoxin was excessive in comparison to the test dose of blood cells; and the amount of hemoglobin and of stroma was derived from only half the amount of blood cells used in the test itself. It is obvious, however, that the inhibition of hemolysis by pneumococcus hemotoxin is due principally to the decrease in effective hemotoxin by its combination with the stroma; and that after the hemotoxin has once combined with the blood cell, hemolysis proceeds even if there is present in the system an amount of stroma which could combine with practically all the hemotoxin if it were free in solution.² It is possible, of course, that the presence of either hemoglobin or stroma does have some influence upon the rate of hemolysis of combined cells, but if there is such an effect it is slight in comparison to the effects due to the combination of the stroma with free hemotoxin. It is also possible that hemoglobin itself, although not evidenced in our experiment, can combine with free hemotoxin, for hemoglobin is said to exist in large molecular aggregates which might have marked absorbing properties.

² The stroma used in our experiments was obtained by distilled H₂O hemolysis and had not been combined with hemotoxin previous to its addition to the above test systems. While it is probable that stroma previously combined with small amounts of hemotoxin would have less combining capacity due to its partial "saturation," the difference in combining capacity would be only a quantitative one that does not influence the conclusions drawn from the present experiment.

Dissociation of Free Hemotoxin during the Hemolysis of Erythrocytes Which Had Previously Combined with an Excess of Hemotoxin.

Preceding experiments have shown that it is possible to combine more hemotoxin with erythrocytes than is required for their hemolysis. It was of interest therefore, to determine whether or not active hemotoxin is set free from the hemotoxin-erythrocyte combination during or after the hemolysis of erythrocytes which have previously been combined with an excess of hemotoxin. A number of experiments were made to investigate this question.

It is obvious from the principles established in preceding experiments, that there are a number of opposing factors that make it difficult to obtain the ideal conditions to demonstrate definitely the dissociation of active hemotoxin. If one combines erythrocytes with too great an excess of hemotoxin, hemolysis is so rapid even at 0°C. that it is difficult to separate the absorbed cells by centrifugation without some hemolysis in the supernatant fluids. In the procedure finally adopted, the dose of hemotoxin was sufficient to cause the hemolysis of more than 40 times the number of blood cells used for absorption and the period of absorption was limited to 5 minutes plus the 5 minutes required for the centrifugation of the mixtures in the ice box. The hemotoxin solution was put into balanced tubes before the addition of the blood cells, and centrifuged 5 minutes afterwards. The absorbed or combined cells were rinsed several times with cold salt solution and the rinsing fluids were always proved free of detectable traces of hemotoxin. After the hemolysis of the combined cells when resuspended in fresh salt solution, a small measured amount of fresh erythrocytes was added to determine whether or not any active hemotoxin had been dissociated during the hemolysis of the combined cells.

The results of these experiments proved that a certain amount of free hemotoxin is dissociated during the hemolysis of erythrocytes which have previously combined with a great excess of hemotoxin. The conditions of the experiment were severe since the combined cells before their resuspension in fresh salt solution had been rinsed free of any possible traces of hemotoxin other than that combined with the erythrocytes; and in that respect, the demonstration of hemotoxin dissociation was conclusive. From a quantitative standpoint, however, the amount set free was extremely slight and practically all the hemotoxin was "consumed" during the hemolysis of a number of blood cells less than 1/40 the hemolyzing capacity of the test dose of hemotoxin. This is an interesting fact for if the hemotoxin acted as enzyme or catalyst, one would expect more of it to be freed in an active form if originally combined in great excess with its substrate.

Effect of Fractional Addition of Erythrocytes upon the Amount of Hemoglobin Liberated by the Action of a Constant Dose of the Hemotoxin.

In the preceding experiment it was found that a certain small amount of active hemotoxin is set free during the hemolysis of erythrocytes which had previously combined with an excess of hemotoxin, but that most of the hemotoxin is consumed by combination with the stroma. From these facts, it seemed that the fractional addition of the blood cells should influence the amount of hemoglobin which can be set free by the action of a constant amount of hemotoxin. While, in one sense, the preceding experiment dealt with the effect of fractional addition of the blood cells, the conditions were severe in that the combined blood cells were separated from the absorption mixture, resuspended in salt solution, and the second lot of blood cells not added until after the hemolysis of the combined cells. The following experiment on the question of the influence of fractional addition differed from the preceding experiment in that the second portion of the blood cells was added to the hemotoxin solution before hemolysis of the first portion was complete.

Since fractional addition of the blood cells was expected to have at least some effect, the test was made more severe by employing a dose of hemotoxin sufficient to cause complete hemolysis of twice the test dose of erythrocytes. The total final amount of the erythrocytes was kept constant, and the differences in the tests consisted only in the order of addition of the blood cells.

The constant dose of hemotoxin was placed in a series of tubes held in an ice box at 0°C. The following amounts of blood cells were then added to separate tubes of the hemotoxin solution: Tube 1: 0.4 cc. (the complete test dose of erythrocytes); Tube 2: 0.2 cc. (one-half the test dose); Tube 3: 0.04 cc. (one-tenth the test dose). Special precautions were taken to insure prompt and uniform mixing in order that all the blood cells might have equal opportunities to combine with hemotoxin. These mixtures were held for 4 hours at 0°C. At the end of this period, the second portions (0.2 and 0.36 cc.) of the test dose of erythrocytes were added to the tubes containing 0.2 cc. and 0.04 cc. of red blood cells; hemolysis was not complete in any of the tubes at the time of the second addition of the blood cells. The mixtures, now containing the same total amount of blood cells, were held an additional 4 hours at 0°C., and were then placed in a water bath at 25°C. for 1 hour. At the end of this period of incubation, the temperature was raised to 37°C. and an additional hour allowed for the complete end-point of the reaction.

The per cent of complete hemolysis was determined by colorimetric examination of the supernatant fluids after centrifugation. In order to avoid errors due to the presence of methemoglobin, the colorimetric measurements were done by the cyanhemoglobin method (8). The results are given in Table IV.

The results of this experiment (Table IV) showed that with a constant dose of hemotoxin, less hemoglobin is liberated from the same number of blood cells when the blood cells are added fractionally than if all of them are added at the same time. This apparent reduction in the hemolyzing capacity of the hemotoxin can be explained by the principles demonstrated in preceding experiments. It has been shown that the hemotoxin distributes itself more or less uniformly upon the blood cells present in the system, and that much more hemotoxin can combine with each erythrocyte than is required for the liberation of all its hemoglobin. Consequently, in experiments of this type, when the first fraction of the total blood cells is added,

TABLE IV.

Effect of Fractional Addition of Erythrocytes upon the Amount of Hemoglobin Liberated by the Action of a Constant Dose of the Hemotoxin.

Order of addition of erythrocytes to the hemotoxin solution	Total amount of erythrocytes	Per cent total hemoglobin set free
	cc.	
All of erythrocytes added at once	0.4	100
Erythrocytes added in two equal portions; first half at once, and second half added later	0.4	87
Erythrocytes added in two unequal portions; 10 per cent added at once, and remaining 90 per cent added later	0.4	53

all the hemotoxin will combine with these cells, provided that precautions (thorough and prompt mixing) are taken to bring all the cells into immediate contact with the hemotoxin. The first fraction of the cells will then hemolyze due to the action of the combined hemotoxin, but during the process most of the hemotoxin will be consumed by combination with the stroma. When the second fraction of the cells is added, their hemolysis will depend upon the free hemotoxin which has dissociated from the hemotoxin-erythrocyte combination during the hemolysis of the combined cells; and the preceding experiment has already shown that only a small portion of the originally effective hemotoxin escapes from combination with the stroma. Quite different conditions obtain when all the blood

cells are added at the same time, for then each of the blood cells has an equal opportunity to combine with the available hemotoxin. Under these conditions, where the concentration of effective hemotoxin is known to be in excess of that required for liberation of all the hemoglobin, one can always expect a constant amount of hemotoxin to set free more hemoglobin than if the blood cells are added fractionally. It is obvious that this relation must be limited to hemotoxin concentrations which are known to be in excess of that required for hemolysis of the total test dose of the blood cells.

It is also obvious that a number of factors can influence the relative or quantitative effects of the fractional addition of the blood cells. One of the most important factors is the proportion of the total blood cells added in the first absorption. Other important factors are the temperature of the system and the time interval between the fractional additions of the blood cells. It is probably especially important whether or not hemolysis of the combined cells has occurred before addition of the second lot of erythrocytes. If the fresh cells are added to the system before hemolysis, the second fraction of cells may be able to dissociate some of the hemotoxin from the erythrocytes previously combined with an excess of hemotoxin. On the other hand, if hemolysis of the first lot of cells is complete, the second lot of cells must depend for their hemolysis upon the hemotoxin which has not combined with the products of hemolysis. In other words, in the first set of conditions, the fresh blood cells compete with unhemolyzed cells combined with an excess of hemotoxin, and in the second set of conditions, they compete with the stroma and other products of hemolysis; and it may be much more difficult to dissociate active hemotoxin from the combination of hemotoxin with the products of hemolysis than from the combination of hemotoxin with the unhemolyzed erythrocytes.

DISCUSSION.

The experiments presented in this paper have dealt with the combination of pneumococcus hemotoxin with the erythrocyte, which is the first step in the hemolytic process.

It was known (3) that the active or reduced form of the hemotoxin combined with the blood cell before it caused hemolysis. The present study of the combining properties of the hemolytically inactive modifications of the hemotoxin was limited to the reversible inactive oxidized form. The results indicated that the reversibly oxidized form of the hemotoxin exists in two different modifications; one retains the property of combining with the blood cell although it has lost the

property of causing the usual hemolysis; the other possesses neither the property of combining with the erythrocyte nor the property of hemolysis proper.

The existence of these two different hemolytically inactive modifications of the reversibly oxidized hemotoxin indicates that the oxidative inactivation of the hemotoxin involves a series of different stages which probably proceed in the following order: Reduced or hemolytically active form \rightarrow Reversibly oxidized modification which retains the erythrocyte-combining property but not the actual hemolytic property \rightarrow Reversibly oxidized modification which possesses neither the combining nor the hemolytic property \rightarrow Irreversible and inactive products. A series of different reactions yielding different intermediate products which is common in biological oxidations is probably a frequent occurrence when antigenic substances are oxidized. Their complex structure and large molecular weight indicate the existence of a large number of molecular groupings, and the successive oxidation of different individual groupings might be expected to yield a series of different products possessing somewhat different properties.

In the previous study (1) it was shown that the reversibly oxidized form of the hemotoxin, although hemolytically inactive, possessed antigenic properties identical with those of the original reduced form of the antigen. These results proved that the hemolytic property can be lost without an accompanying loss of the antigenic properties. At the time of the investigation of the antigenic properties, the existence of the two different modifications included in the reversibly oxidized form of the hemotoxin had not been recognized. However, it is logical to believe that the antigenic properties assigned in the previous paper to the reversibly oxidized hemotoxin are certainly possessed by the modification which retains the combining property, for it represented the greater¹¹ proportion of the total reversibly oxidized hemotoxin contained in the oxidized solutions used in the immunization. The antigenic properties of the second reversibly oxidized modification which has lost the combining property remains an unsettled question.

The combining properties of the different modifications of pneumococcus hemotoxin are of immunological interest from a general point

of view. The most satisfying proof of the function of different molecular groupings in the combining and injury reactions of hemotoxins has been based upon the separation of the erythrocyte-combining reaction from the erythrocyte-hemolyzing reaction by means of the absorption of the active hemotoxin in the cold. Without a means of detecting the inactive forms themselves, it has been impossible to determine whether or not inactive hemotoxoids retain the erythrocyte-combining property (the so called "haptophore" group) in spite of the loss of the erythrocyte-hemolyzing property (the so called "toxophore" group). While we did not succeed in causing hemolysis by reduction treatment of the blood cells combined with the oxidized lysin, the application of the reduction technic to the supernatant fluids of the absorbed mixtures proved that a large proportion of the reversibly oxidized, inactive form was removed by combination with erythrocytes in the cold. This constitutes more direct evidence than has hitherto been obtained of the existence of a hemotoxin modification which has lost the property of hemolysis proper without loss of the property of combining with the blood cell. Although no antigenic properties can yet be assigned to the other reversibly oxidized modification of pneumococcus hemotoxin which has lost the combining property as well as the hemolytic property, it too is of interest. The fact that reduction treatment can restore both of the properties lost by oxidation is indicative of the possibility of changing a variety of different properties of complex antigenic substances without inducing profound and irreversible changes in the molecule as a whole.

A number of other experiments were made upon the combining properties of the reduced or active form of the hemotoxin which, because of the advantage of a separation of the reactions of combination and hemolysis proper, are of interest in connection with the mechanism of the hemolytic action of pneumococcus hemotoxin. (All these experiments were conducted with excess doses of hemotoxin and the results cannot safely be applied to systems where the hemotoxin concentration is less than that required for complete hemolysis.) Evidence was obtained that with a constant concentration of hemotoxin, the number of hemotoxin molecules which combine with the individual erythrocytes is inversely related to the blood cell concen-

tration; and that the rate of the actual hemolysis is directly related to the number of hemotoxin molecules which have previously combined with the individual erythrocytes. The same experiments also proved that a constant dose of hemotoxin can be removed by an amount of blood cells much less than the amount which the hemotoxin can hemolyze. Tests with rabbit cell stroma showed that the absorbing or combining capacity of the stroma derived by distilled water hemolysis is approximately equivalent to that of the intact red cells themselves.

Experiments were also made to determine the inhibitory effect of stroma and of dissolved hemoglobin upon the two steps (combination and hemolysis proper) involved in the hemolytic action of hemotoxin. The results showed that the final inhibition of pneumococcus hemotoxin hemolysis is due principally to the removal of effective hemotoxin by combination with the stroma. After the hemotoxin had once combined with the blood cell, the hemolysis proceeded even when the amount of stroma present in the system would have been sufficient to combine with all the hemotoxin had it been free in the solution. It was also found that some free hemotoxin is dissociated during the hemolysis of erythrocytes which have previously combined with an excess of hemotoxin, but the amount set free is surprisingly small for most of it is consumed by combination with products of the hemolytic reaction, especially with the stroma. While this apparent "using up" of hemotoxin during the hemolytic reaction is somewhat similar to the combination of enzymes with their reaction products, the combining property of the stroma is probably of a higher order of magnitude than that of the reaction products of the common enzymes, and consequently, causes a correspondingly greater inhibitory effect. However, the fact that such a great proportion of the hemotoxin is consumed when large excesses of hemotoxin are combined with small numbers of blood cells, is an argument against the enzymatic nature of its hemolytic action.

The fractional addition of red blood cells decreases the hemolyzing "capacity" of a constant dose of hemotoxin; a dose of hemotoxin more than sufficient to cause the hemolysis of the test dose of blood cells when all were added at once, was unable to cause complete hemolysis when the same number of erythrocytes was added fractionally. This

agrees with the previously established relationships and is probably due to the inhibition of the hemotoxin by its combination with the stroma. The combination reaction proceeds so rapidly, even at 0°C., that in order to obtain the maximum liberation of hemoglobin, the absorption mixtures must be immediately and thoroughly shaken in order to give all the erythrocytes an equal opportunity to combine with the available hemotoxin.

SUMMARY.

The investigation dealt particularly with the combination of pneumococcus hemotoxin with red blood cells. The reduced or hemolytically active form of the hemotoxin was known to possess the property of erythrocyte combination. The results obtained in this study indicate that the hemolytically inactive, reversibly oxidized form exists in two different modifications; one retains the property of erythrocyte combination while the other lacks this property.

The influence of the combining property of the hemotoxin upon the mechanism of hemolysis and upon the inhibition of the hemolytic reaction was also investigated.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

X. IMMUNOLOGICAL DISTINCTIONS BETWEEN THE HEMOTOXIN AND THE "PROTEIN FRACTION" OF THE PNEUMOCOCCUS CELL.

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INTRODUCTION.

It is known (1, 2) that the hemotoxin and the "protein fraction"¹ of the pneumococcus cell possess the following properties in common: (1) both are cellular constituents of *Pneumococcus* and are present in solutions prepared from pneumococcus cells; (2) both react with species-specific, rather than type-specific antibodies; (3) antibodies (antihemotoxin and precipitins) reactive with both of them are present in immune serum produced by injection of solutions of pneumococcus cells. The object of the present paper is to determine whether or not they represent distinct and separate antigenic constituents of *Pneu-*

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¹ The term "protein" or "protein fraction" of the pneumococcus cell is limited in this paper to the protein substances which react with the species-specific anti-protein precipitins which are present in immune serum produced by immunization with solutions of pneumococcus cells (3, 4). These substances, as pointed out by Avery and Heidelberger (5, 6), include the bacterial proteins (mainly nucleoprotein and mucoid) which are precipitated in the cold by acetic acid, and thus may include a number of separate but unrecognized antigens which give rise to individual antiprotein precipitins. For clearness of expression in this paper, it is desirable to use the term "protein" in the inclusive sense of Avery and Heidelberger (6) although it is probable that the hemotoxin itself, like all true antigens, is also protein in nature; as a matter of fact, it is also precipitated, at least in part, by the acetic acid treatment since the reversible oxidation products of the hemotoxin are demonstrable in some solutions of pneumococcus "protein" (7).

mococcus, which are immunologically reactive with distinct and separate antibodies.

The presence of protein precipitins in immune sera is a factor always to be considered in connection with the neutralization of primarily toxic antigens, and a distinction between the antibody which neutralizes the hemotoxin and other antibodies present in the serum is particularly important in view of the fact that the antihemotoxin can be produced by immunization with hemolytically inactive forms of the hemotoxin (2, 8). Hence, the immediate object of this study is to establish pneumococcus hemotoxin as an integral antigenic substance in order to show that the preceding reports (2, 8, 9) had dealt with a true antigen. However, proof of the individuality of the hemotoxin is also of interest from a more general point of view since it adds another definite antigen to the list of constituents included in the "antigen mosaic" of the pneumococcus cell.

EXPERIMENTAL.

Methods.—The methods were essentially the same as described in preceding papers (2, 8, 9).

Differences in the Effect of Previous Exposure to 55°C. upon the Antibody-Invoking Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

The following experiment dealt with the difference in the effect of previous exposure to 55°C. upon the antibody-invoking properties of the hemotoxin and of the "protein" fraction of Pneumococcus. One series of rabbits was immunized with bacterial extract which had been heated at 55°C. for 10 minutes, a treatment which previous experiments (8) had indicated was sufficient to destroy the antibody-invoking property of the hemotoxin; a control series was immunized with the unheated extract. In order to make the comparison more convincing, different amounts of pneumococcus cell solution were injected into corresponding animals in the two series and the immunization of some animals was carried over a long period. Bleedings were made after each course of injections, and the sera tested for antihemotoxin and antiprotein precipitin. The objects of the experiment are satisfied by presenting in Table I the results obtained with the animals immunized with 0.2 cc. doses of the pneumococcus solution.

The results (Table I) of this experiment reveal a distinct difference in the effect of short exposure to 55°C. upon the antibody-invoking

properties of the hemotoxin and the "protein fraction" of *Pneumococcus*. The heating treatment only slightly weakened the effective-

TABLE I.

Influence of Exposure to 55°C. upon the Antibody-Invoking Properties of Pneumococcus Hemotoxin and of Pneumococcus "Protein."

Animals immunized with pneumococcus cell solution	Serum	Antihemotoxin ¹		Antiprotein precipitins ²	
		Hemolysis by 5 "units" of hemotoxin, which had been incubated in presence of serum before addition of blood cells		Precipitation of protein from solutions of a heterologous type of pneumococci	
		Amount of serum, cc.		Dilution of antigen	
		0.05	0.01	1/10	1/50
Unheated	Normal serum; before immunization	****	****	0	0
	Immune serum; after 1 course of 6 injections of 0.2 cc. of the bacterial solution	0	*	++	+
	Immune serum; after 6 courses of 6 injections of 0.2 cc. of the bacterial solution	0	0	+++	+++
Heated 10 min. at 55°C.	Normal serum; before immunization	****	****	0	0
	Immune serum; after 1 course of 6 injections of 0.2 cc. of the bacterial solution	****	****	+	0
	Immune serum; after 6 courses of 6 injections of 0.2 cc. of the bacterial solution	****	****	+++	+++

¹0 = no hemolysis.

* = hemolysis approximately one-fourth complete.

** = hemolysis approximately one-half complete.

*** = hemolysis approximately three-fourths complete.

**** = hemolysis complete.

²0 = no detectable reaction.

+

++ = moderate cloudiness.

+++ = heavy cloud, with large amount of precipitate in bottom of tube.

ness of the antigen (or antigens) related to the antiprotein precipitin; but completely destroyed the antibody-invoking property of the hemo-

toxin. A quantitative loss in the antigenic capacity of the heated "protein" was evident throughout the experiment; *i.e.*, either a larger amount of the bacterial solution, or a more prolonged immunization was always required with the heated than with the unheated bacterial solution in order to obtain a serum of the same antiprotein precipitin content. However, these quantitative differences became less as the immunization was continued, and (as shown in Table I) after several courses of injections the sera obtained by use of the heated (55°C. for 10 minutes) bacterial extract were equal in antiprotein precipitating power to those obtained by injection of the unheated material.

Quite different relations obtained with the hemotoxin. In agreement with the results of a previous investigation (8) the heating treatment seemed to result in an absolute loss of its antibody-invoking property, for even after prolonged immunization, no antihemotoxin could be detected in the serum of animals injected with the heated pneumococcus solutions. This distinction between the effect of heat upon the antibody-invoking properties of the hemotoxin and the "protein fraction" is particularly convincing in view of the quantitative control of the amount of material employed in the immunizations.

Differences in the Influence of Previous Exposure to 55°C. upon the Antibody-Combining Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

That the hemotoxin completely loses its antibody-combining property when heated at 55°C. for 10 minutes has been shown in a previous paper,² no antihemotoxin being combined when heated pneumococcus hemotoxin was added to immune serum. A number of experiments were made to determine whether or not the same heating treatment affects the antibody-combining property of the protein fraction. The pneumococcus cell solution was heated for 10 minutes at 55°C. without causing any visible clouding or precipitation in the clear solution; as far as we could determine this heated solution (in which the antibody-combining property of the hemotoxin had been destroyed) was precipitated by antiprotein serum to exactly the same degree as was unheated pneumococcus solution in parallel titrations against the same immune serum.

From the results of the above experiment, it is evident that exposure of a solution containing both hemotoxin and "protein" to a tempera-

² Neill, Fleming, and Gaspari (8), Table IV.

ture of 55°C. for 10 minutes inactivates the antibody-combining property as well as the antibody-invoking property of the hemotoxin, but destroys neither of these properties of the "protein." Since these two properties are the fundamental properties of all true antigens, the loss of both of them by the hemotoxin and the loss of neither by the "protein" indicate that although contained in the same bacterial solution, the hemotoxin represents an antigenic constituent distinct from those which give rise to the antiprotein precipitins.

Differences in the Influence of Treatment with High Concentrations of H_2O_2 upon the Antigenic Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

A distinction between the hemotoxin and the protein fraction was also made upon the basis of the effect of treatment with high concentrations of H_2O_2 upon their antigenic properties. It was found that treatment with this oxidizing agent which destroyed the antigenic properties of the hemotoxin (8) did not inactivate the protein fraction; the treated solutions, although containing no antigenically effective hemotoxin, still retained both the *in vivo* property of invoking the production of antiprotein precipitins and the *in vitro* property of reacting with antiprotein precipitins.

Production of Immune Serum Containing Antiprotein Precipitins but No Antihemotoxin by Immunization with Pneumococcus Cell Solutions from Which the Hemotoxin Had Been Removed by Absorption with Red Blood Cells.

The experiments consisted of a comparison of the antihemotoxin and antiprotein precipitin content of the immune serum obtained from two series of rabbits: (1) the first series, immunized with "unabsorbed" pneumococcus cell solution containing both the hemotoxin and the "protein fraction;" (2) the second series, immunized with the same amounts of pneumococcus cell solution from which the hemotoxin had been removed by previous absorption.

Immunization.—Each animal in the two series received six daily subcutaneous injections of 0.1 cc. of the "absorbed" or "unabsorbed" pneumococcus solution. To avoid the possibility of producing isohemolysins or isoagglutinins in the series immunized with the "absorbed" material, the solution used for the immunization of each rabbit was kept separate and absorbed with the cells from its own blood. The blood cells were washed before each absorption from a stock of the normal

defibrinated blood of each animal obtained before immunization. The absorptions were carried out as described for previous experiments. The material for one course of injections was absorbed with blood-cells at the beginning of the experiment; a second absorption was carried out every other day on quantities of the previously absorbed solution sufficient for two injections. The animals were bled after each course of injections, and the immune serum titrated for antihemotoxin and antiprotein precipitins by the previous methods.

TABLE II.

Antihemotoxin and Antiprotein Precipitins in Serum of Animals Immunized with "Absorbed" and "Unabsorbed" Pneumococcus Cell Solutions.

Immunization material	Rabbit	Antihemotoxin titration ¹ (hemolysis by 5 units of hemotoxin which had been incubated in presence of serum before addition of blood cells)		Antiprotein precipitin ² titration	
		Amount of serum, cc.		Dilution of antigen	
		0.06	0.01	1/2	1/10
		Hemolysis	Hemolysis	Precipitation	Precipitation
"Absorbed" with erythrocytes	1	***	****	++	+
	2	****	****	++	+
Not "absorbed" with erythrocytes	3	0	*	++	+
	4	0	*	++	+

¹0 = no hemolysis.

* = hemolysis approximately one-fourth complete.

** = hemolysis approximately one-half complete.

*** = hemolysis approximately three-fourths complete.

**** = hemolysis complete.

²0 = no detectable reaction.

+

++ = moderate cloudiness.

+++ = heavy cloud, with large amount of precipitate in bottom of tube.

The results of the tests of the sera after one course of injections are presented in Table II.

The results (Table II) of this experiment show that the absorption of pneumococcus cell solution with erythrocytes removed the antigen responsible for antihemotoxin production, but was without effect upon

the antigens responsible for the production of the antiprotein precipitins. Immunization with pneumococcus cell solutions which had been absorbed with red blood cells yielded the same type of serum as did immunization with pneumococcus solutions which had been heated at 55°C.; *i.e.*, an immune serum containing antiprotein precipitins but no antihemotoxin.³ The fact that it is possible to obtain an immune serum containing only one of the antibodies (the antiprotein precipitin) is due in both cases to the loss of the other antigen (hemotoxin) which is effective in the original pneumococcus solution, the hemotoxin antigen being lost in the first instance (Table I) by heat inactivation and in the second instance (Table II) by removal through combination with erythrocytes.

*Effect of Removal of the Hemotoxin upon the Protein Precipitation
Reaction of Pneumococcus Cell Solutions with Antiprotein
Immune Serum.*

This experiment dealt with the effect of the removal of the hemotoxin upon the protein precipitation reaction of pneumococcus cell solutions with antiprotein immune serum. Parallel antiprotein precipitin titrations were made with immune antiprotein serum, (1) against "unabsorbed" pneumococcus cell solution (containing both the hemotoxin and the "protein fraction"); and (2) against "absorbed" pneumococcus cell solution (from which the hemotoxin had been removed by previous absorption with red blood cells).

The hemotoxin was absorbed from the pneumococcus solution by the procedure employed in previous experiments. The "absorbed" bacterial solution and "unabsorbed" bacterial solution were then tested against immune serum (obtained by immunization with heated pneumococcus solution) which contained antiprotein precipitins but no antihemotoxin. The protein precipitin tests were made with a constant dilution of serum (2/5) against two dilutions (1/10 and 1/20)

³ The separation of the hemotoxin by absorption with erythrocytes is apparently not so absolute as that obtained by heating the bacterial solutions. With pneumococcus solutions in which the hemotoxin has been inactivated by heat, no trace of antihemotoxin was produced even after prolonged immunization. In our experiments with "absorbed" solutions, slight traces of antihemotoxin were produced by one of the animals when the immunization was continued. We believe, however, that this was probably due to traces of hemotoxin remaining in the "absorbed" solution which, while insufficient to induce a definite antigenic response to the one course of injections reported in Table III, were sufficient to cause a weak response when a large number of injections were given.

of the bacterial solution. Controls of the bacterial solutions were prepared with normal serum instead of salt solution, since the "absorbed" bacterial extract became cloudy if too greatly diluted with salt solution. The protocol is presented in Table III.

The results (Table III) show that removal of the hemotoxin had no detectable effect upon the protein precipitation reaction of pneumococcus cell solutions when tested against antiprotein immune serum. While this fact in itself is not proof that the hemotoxin is a distinct

TABLE III.

Effect of Removal of the Hemotoxin upon the Protein Precipitation Reaction of Pneumococcus Cell Solutions with Antiprotein Immune Serum.

Pneumococcus solution	Protein reactive with anti-protein immune serum ¹		Hemotoxin ²	
	Dilution of antigen		Amount of bacterial solution, cc.	
	1/10	1/20	0.10	0.01
	Precipitation	Precipitation	Hemolysis	Hemolysis
"Absorbed" with red blood cells	++	+	0	0
"Unabsorbed"	++	+	****	****

¹0 = no detectable reaction.

+ = faint cloudiness.

++ = moderate cloudiness.

+++ = heavy cloud, with large amount of precipitate in bottom of tube.

²0 = no hemolysis.

* = hemolysis approximately one-fourth complete.

** = hemolysis approximately one-half complete.

*** = hemolysis approximately three-fourths complete.

**** = hemolysis complete.

antigenic entity, it is evidence that the hemotoxin in "unabsorbed" solutions, if it reacts at all with the antiprotein precipitin, does not constitute a significant portion of the material precipitated by the precipitins in antiprotein immune serum.

The Occurrence of Antihemotoxin and Antiprotein Precipitins in Antipneumococcus Diagnostic and Therapeutic Serum from Immune Horses.

The antipneumococcus serum usually employed for typing and for therapeutic purposes is obtained from horses instead of from rabbits. It was of interest,

therefore, to determine whether or not the relations established with rabbit immune serum hold true for serum obtained from immune horses. Horses are usually immunized with suspensions of the bacterial cells instead of with the filtered solutions employed in the previous experiments. However, as Avery and Heidelberger (6) have pointed out, these pneumococcus suspensions consist of a mixture of the type-specific antigen and of the species-specific protein which gives rise to the antiprotein precipitin. Hence, the usual antipneumococcus serum from immune horses contains in addition to the type-specific antibody, varying amounts of the species-specific antiprotein precipitin.

The antihemotoxin, like the antiprotein precipitin, is produced in response to an endocellular antigen, and hence, is likewise present in the usual antipneumococcus horse serum. Provided the strain employed in the immunization was not a poor hemotoxin producer, one would expect a certain parallelism between the antiprotein precipitins and antihemotoxin, since the production of both of them would be enhanced by the presence of a large number of autolyzed pneumococci and the continued injection of large amounts of material. As a matter of fact, most antipneumococcus horse serum does contain much more antihemotoxin and antiprotein precipitins than do the usual immune rabbit sera. It seemed that this fact might be due not only to the large amount of material injected and the prolonged immunization, but also to the common use of live pneumococci in the final stages of the immunization.

In connection with experiments on the production of antipneumococcus serum at the Massachusetts Antitoxin and Vaccine Laboratory by Dr. Benjamin White and Dr. Elliott S. Robinson, one series of horses was immunized with heated pneumococcus suspensions alone, and another series with the unheated filtrate of a frozen and thawed suspension of pneumococci in addition to the heated bacteria. Samples of different bleedings of these two sorts of antipneumococcus sera furnished to us by Dr. White and Dr. Robinson, were employed in the experiments. In addition to the sera from the Massachusetts State Laboratory, two antipneumococcus horse sera from other laboratories which employ live bacteria were included.

The experiments consisted of comparisons of the antihemotoxin content of antipneumococcus horse serum produced by immunization with three sorts of material: (1) heated suspensions of pneumococci; (2) heated pneumococci plus unheated filtrate of dissolved pneumococci; (3) heated pneumococci followed by injections of live pneumococci. The sera included Type I serum prepared for therapeutic purposes, and Type III serum prepared for diagnostic typing; no Type II sera were used because of the type-specific carbohydrate precipitation which would have occurred in the (Type II) pneumococcus solution used as the source of the hemotoxin in the experiment proper. (The antihemotoxin is not type-specific and the use of a heterologous type of pneumococcus solution in the antihemotoxin test of the serum is to be preferred.)

A summarized protocol is presented in Table IV.

The results (Table IV) of the tests of the antipneumococcus serum from horses are in agreement with the previous results with immune rabbit serum. Like the rabbit serum obtained after immunization with heated pneumococcus solution, the horse serum obtained by immunization with heated pneumococci alone contained no antihemotoxin; the slight inhibition by the largest amount of serum (0.05

TABLE IV.

Antihemotoxin in Serum of Horses Immunized with Heated and Unheated Pneumococci.

Antipneumococcus serum from immune horses	Material employed in immunization	Antihemotoxin titrations ¹			
		Hemolysis by 5 units of hemotoxin which had been incubated in the presence of serum before addition of the blood cells			
		Amount of serum, cc.			
		0.05	0.01	0.007	0.003
Type I (therapeutic)	Heated pneumococcus cells (vaccine)	***	****	****	****
Type I (therapeutic)	“ “ “ “	***	****	****	****
Type I (therapeutic)	“ “ “ plus unheated pneumococcus solution	0	0	0	0
Type I (therapeutic)	Heated pneumococcus cells plus unheated pneumococcus cells	0	0	0	0
Type III (diagnostic)	“ “	0	*	**	****

¹0 = no hemolysis.

* = hemolysis approximately one-fourth complete.

** = hemolysis approximately one-half complete.

*** = hemolysis approximately three-fourths complete.

**** = hemolysis complete.

cc.) is no more than that exhibited by normal horse serum and is undoubtedly due to the non-specific action of the lipid constituents which are unrelated to the immunological antihemotoxin (2). In contrast, the horse sera obtained by immunization with either unheated solutions of pneumococci or with the live bacteria, contained large amounts of the specific antihemotoxin.

All the same sera also contained significant amounts of antiprotein

precipitins, the relative amounts of the precipitins in each serum being roughly parallel to its content of hemotoxin. A certain relation between the content of antihemotoxin and antiprotein precipitins is to be expected in antipneumococcus serum, since the heating treatment which causes a complete loss of the effectiveness of the hemotoxin antigen also causes a quantitative diminution in the antigenic effectiveness of the protein fraction. (A more complete study of the effect of heat upon the antigenic effectiveness of pneumococcus protein will be presented in a later paper.) However, there is the same evidence of the separation of the hemotoxin from the protein fraction in the serum from horses immunized with heated pneumococci as in the serum from rabbits immunized with heated solutions of the bacterial substances, each of them being devoid of antihemotoxin although containing easily demonstrable antiprotein precipitins.

Separation of the Hemotoxin from the Toxic Substance in Pneumococcus Solutions Which Cause the Acute Death of Rabbits.

Since the present paper deals with the hemotoxin as an integral antigenic constituent of the pneumococcus cell, it is desirable to present evidence that the hemotoxin is distinct from the toxic substances ("endotoxin" (10)) contained in the same pneumococcus solutions. That the presence of active hemotoxin itself was not essential to the toxicity of pneumococcus solutions was indicated by the fact that the hemolytic activity of the solutions could be destroyed either by heat (55°C. for 5 minutes) or by oxidation, without loss in their toxicity. However, it was desired to obtain more direct evidence of the distinction between the hemotoxin and the toxic substances concerned in the acute death of rabbits. The experiments consisted of a comparison of the toxicity of pneumococcus solutions containing the hemotoxin, with the toxicity of the same solutions after removal of the hemotoxin by selective absorption with red blood cells. The comparison was made more valid by using two different doses of each pneumococcus solution: (1) a dose of 0.25 cc. which was not over twice the minimum dose required for the invariable production of acute death in rabbits; (2) a dose of 0.025 cc. which served as a control that the first dose was not an excessive one.

Two series of four rabbits of equal weight were injected intravenously with the "absorbed" and "unabsorbed" pneumococcus solutions. The rabbits which were killed by the injections were autopsied at once by Dr. Arthur Wright of the Department of Pathology. The pathological findings were the same in the animals injected with the "absorbed" solutions (containing no hemotoxin) as in those injected with the "unabsorbed" solutions. The heart was still beating; the lungs were collapsed and not congested; there was marked congestion in the small in-

testine; the bladder wall was completely collapsed. There were never more than a few, if any, slight focal hemorrhages in the lung. The relative absence of hemorrhage or hemolysis was equally evident in the animals injected with the unabsorbed and the absorbed solutions, and was due in all probability to the small doses of bacterial solution employed.

A protocol showing the toxicity and hemolytic activity of the two solutions is given in Table V.

TABLE V.

Separation of the Hemotoxin from the Toxic Substances in Pneumococcus Solutions Which Cause the Acute Death of Rabbits.

Pneumococcus solution	Toxic action (Effects of intravenous injections into rabbits)		Hemolytic action ¹ (Hemolysis of 2.0 cc. of 1 per cent red blood cells)	
	Amount of pneumococcus solution, cc.		Amount of pneumo- coccus solution, cc.	
	0.25	0.025	0.10	0.01
"Absorbed" with red blood cells	Rabbit 1—died 1 hr. 50 min. Rabbit 2—died 1 hr. 10 min.	Rabbit 5—no visi- ble effect Rabbit 6—no vis- ible effect	0	0
"Unabsorbed"	Rabbit 3—died 1 hr. 30 min. Rabbit 4—died 1 hr. 10 min.	Rabbit 7—no vis- ible effect Rabbit 8—no vis- ible effect	****	****

¹0 = no hemolysis.

* = hemolysis approximately one-fourth complete.

** = hemolysis approximately one-half complete.

*** = hemolysis approximately three-fourths complete.

**** = hemolysis complete.

The results (Table V) of these experiments present evidence that different constituents are involved in the hemolytic and toxic activities of solutions of dissolved pneumococci. The solution from which all the hemotoxin had been removed by test-tube absorption with erythrocytes possessed the same degree of toxicity and caused the same gross pathological changes when injected intravenously into rabbits, as did the "unabsorbed" solution which contained the hemotoxin. Since the test doses employed were sufficiently small to detect a significant quantitative diminution in the toxicity of the solutions, one can con-

clude that the hemotoxin is a substance quite distinct from the pneumococcus substances ("endotoxin") which cause the acute, anaphylactoid death of rabbits. Probably the hemotoxin is not involved at all in the toxic action of moderate doses of the usual pneumococcus solutions, for unless excessive doses were injected, the inhibiting action of lipid constituents of normal serum would neutralize a large amount of the hemotoxin.

DISCUSSION.

In a previous paper (2) the true antigenic nature of pneumococcus hemotoxin was indicated by the specificity of its neutralization by an antibody (antihemotoxin) invoked by immunization with solutions of pneumococcus cells; the immunological neutralization was differentiated from the non-specific inhibitory effect of normal serum by evidence that the antibodies in the immune serum did not affect the hemolytic activity of digitonin nor of the hemotoxins of other bacteria. While these facts seemed to establish the hemotoxin as a true antigen which invokes the production of a specific neutralizing antibody, it was desired to differentiate the hemotoxin from the other antigenic substances contained in pneumococcus solutions, and to distinguish the antihemotoxin from the other antibodies contained in the serum produced by immunization with the same bacterial solutions. The most important of the other antigenically effective substances contained in pneumococcus solutions are the protein constituents which give rise to antiprotein precipitins. Proof of the hemotoxin as an individual antigenic constituent of *Pneumococcus*, therefore, requires a satisfactory differentiation of the hemotoxin and the "protein fraction," and a like differentiation of the antihemotoxin and the antiprotein precipitins.

The present investigation has differentiated the two antigens on the basis of the following differences in their properties: (1) The antigenic (antibody-invoking and antibody-combining) properties of the hemotoxin were destroyed by heat and oxidation treatments which did not cause the loss of the antigenic effectiveness of the "protein fraction." (2) Removal of the hemotoxin from pneumococcus solutions by combination with erythrocytes caused no loss in the capacity of the solution to invoke the production of antiprotein precipitins nor in its capacity

to react with antiprotein immune serum. Consequently, immunization with pneumococcus solutions containing both hemotoxin and the "protein fraction" yielded a serum containing both antihemotoxin and antiprotein precipitins, but immunization with solutions which either had received certain heating or oxidation treatment or had been absorbed with red blood cells, yielded a serum containing only antiprotein precipitins and no antihemotoxin. If one accepts the fundamental principle that one antigen gives rise to one antibody, the production of an immune serum containing the usual titre of antiprotein precipitins but no antihemotoxin is in itself evidence that the hemotoxin is an integral entity which gives rise to a specific antibody (antihemotoxin) distinct from the antiprotein precipitin invoked by other antigenic constituents of the pneumococcus cell.

The evidence obtained in a study of different antipneumococcus sera from immune horses agreed with that obtained from experiments on rabbits, and indicated that the injection of either unheated solutions of pneumococci or of unheated suspensions of pneumococcus cells will always yield an immune serum containing relatively large amounts of the antihemotoxin; while the injection of heated solutions or heated suspensions will yield a serum containing little, if any, of the antihemotoxin antibody.⁴

The establishment of the hemotoxin as a distinct antigen is of further interest as an additional example of the variety of separate and distinct antigenic substances contained within a single bacterial cell. It is now recognized that the presence of a number of different antibodies contained in an antibacterial serum is due to the presence of a number of different antigenic substances in the material injected into the animals, each antigen in all probability giving rise to its own antibody. From Avery and Heidelberger's work (6) the presence of the two most important antibodies (the type-specific anticarbohydrate precipitin and the species-specific antiprotein precipitin) in antipneumococcus serum can be assigned to the presence of two distinct antigens in the

⁴ While in our experiments the heating treatment of the immunization material seemed to be the most important factor, the antihemotoxin content of the immune serum would also be affected by variations in the hemotoxin-producing capacities of different strains of pneumococci, amount of material injected, duration of immunization, and differences in the response of individual animals

pneumococcus cell. Due to the above differentiation of the antihemotoxin from the antiprotein precipitin, it is now possible to assign a third type of antibody (antihemotoxin) which is present in most anti-pneumococcus sera, to another separate and distinct constituent (hemotoxin) of the pneumococcus cell.

It is desirable to point out, however, that the evidence has simply established the hemotoxin as an individual antigenic entity; and in spite of the described differences in the effect of certain heating and oxidative treatments upon their antigenic properties, the hemotoxin and the "protein fraction" may possess other immunological properties in common. From one point of view (8), there is no real proof that the hemotoxin is not also a "precipitinogen" which possesses an active hemolytic property demonstrable in higher dilutions than can be detected by serological precipitation. The "protein fraction" of the pneumococcus cell is known to include a number of different proteins, which are difficult to separate. Each of these proteins, if sufficiently different in chemical structure can be expected to give rise to its own antibody; but none of these different antigens can be distinguished immunologically in the absence of strikingly specific properties comparable to the specific property of the hemotoxin. For example, in spite of the fact that it is an individual antigenic entity, the removal of the hemotoxin from the "protein" was detected only by virtue of its hemolytic property; and neither its removal nor that of any other of the "protein" constituents would be detected by the precipitin reaction if it constituted too small a proportion of the total reactive protein.

SUMMARY.

The investigation deals with the immunological differentiation of the hemotoxin and the "protein fraction" of *Pneumococcus*, and with a like differentiation of the antihemotoxin and the antiprotein precipitins. The distinction is made upon the basis of the following evidence: (1) The antigenic (antibody-invoking and antibody-combining) properties of the hemotoxin were destroyed by heat and oxidation treatments which did not cause the loss of the antigenic effectiveness of the "protein fraction." (2) The removal of the hemotoxin from pneumococcus solutions by combination with erythrocytes caused no loss in

the capacity of the solution to invoke the production of antiprotein precipitins nor in its capacity to react with antiprotein immune serum.

Titration of the antihemotoxin content of antipneumococcus horse serum (both diagnostic and therapeutic) indicated that the heating treatment of the immunization material is the most important factor in determining the antihemotoxin content of the immune sera obtained from horses, as well as of that from rabbits.

A distinction was also made between the hemotoxin and the toxic substances ("endotoxin") which cause the acute anaphylactoid death of rabbits.

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A COMPARATIVE STUDY OF SMOOTH AND ROUGH PNEUMOCOCCUS COLONIES.

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PLATES 31 AND 32.

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INTRODUCTION.

Our knowledge of the production of variant pneumococcus forms has been greatly clarified during the last few years by recognition of the fact that the species may be divided into at least two great groups including the so called S, or smooth forms, and the R, or rough forms. Although the terms rough and smooth have been derived essentially from the morphological characteristics of the colonies formed by respective organisms of each group, they also bear a significant relationship to virulence, serological, and other properties exhibited by the organism. This general concept of bacterial dissociation into rough, smooth, and possibly other groups has not only facilitated the classification of pneumococcus variants but also those of other forms. It has found application in a great variety of bacterial species and, as has recently been emphasized by Hadley's review (1), has become of fundamental importance in bacteriology.

With the pneumococcus a number of features have been recognized as characteristic of the R strains, serving to differentiate them from the S or parent strains. Primarily the surface colonies formed by R strains are atypical, presenting a rough surface in contrast to the typical smooth surfaced colonies of the S forms (2-6). Other recognized properties of the R pneumococcus include those of avirulence for mice,* loss of capsule, inability to produce the so called pneumo-

* The definition of avirulence has in this instance been based upon the fact that 1 cc. of an early broth culture when injected into the peritoneal cavity of the mouse fails to kill.

coccus soluble specific substance and consequent loss of type specificity (7), and an increased resistance to the lytic action of bile (8). Fairly extensive studies have been made on the immunological and other properties of the R pneumococcus, notably those of Reimann (5, 7); the colonies, however, have received more limited attention, and, as our ability to recognize the R forms is in some measure dependent upon the appearance of the colonies which they produce, we have turned our attention to this phase.

It is quite evident at the outset that such a study presents a number of variable factors in that bacterial colonies are structures of somewhat labile nature particularly in the case of an organism such as the pneumococcus, which is extremely susceptible to changes in its environment, resulting in a colony expression which may be quite different in different environments. Our colony studies have been consequently restricted to a comparison between those produced by a small number of standard S and R strains observed under a limited number of cultural conditions. We have not included any of the intermediate strains which have been described by Yoshioka (3) and Blake and Trask (4).

Methods.

The smooth or S strains of type-specific pneumococci were obtained in all instances from the sputum of cases of lobar pneumonia which occurred at the Pennsylvania Hospital during the winter of 1926-27. All of them were subjected to one animal passage and have been subsequently kept at room temperature on rabbit blood agar, with weekly transfers.

The rough or R strains of pneumococci, R-I and R-II, were obtained from The Rockefeller Institute for Medical Research, through the kindness of Dr. O. T. Avery. A third strain which we have designated as "R-III" was isolated from a strain of Type III pneumococci which invariably produced rough colonies when plated upon acid media. These strains were kept in a similar manner on rabbit blood agar.

Strains of *Streptococcus viridans* and hemolytic streptococci, used for comparative colony studies, were obtained from recent blood cultures of cases of subacute bacterial endocarditis and hemolytic streptococcus septicemia respectively. In most of the subsequent experiments fourteen strains of organisms have been used, including nine S pneumococcus strains (three of each type), three R pneumococcus strains (R-I, R-II, and "R-III"), one strain of *Streptococcus viridans*, and one of *Streptococcus hemolyticus*.

The anti-S or type-specific antipneumococcus serum employed for the precipi-

tation and agglutination tests was obtained from the Division of Laboratories and Research, New York State Department of Health. Anti-R pneumococcus serum was prepared by the inoculation of rabbits with strains of *Pneumococcus* R-I and R-II according to the method of Cole and Moore (9).

For colony studies we have selected as a standard the surface growth of 24-48 hour plate cultures upon fresh rabbit blood agar (pH 7.8). The colonies have been studied under the low power objective of the microscope by reflected daylight from the tilted surface of the plate. A mercury vapor lamp was used as the light source in making photographs.

The Smooth Pneumococcus Colony.

In order to appreciate or emphasize the differences between S and R pneumococcus colonies it is important to review briefly the characteristics of the former. Colonies produced by virulent pneumococci when grown upon the surface of suitable media have long been recognized as presenting characteristic appearances. More than 20 years ago Buerger (10) described and emphasized their distinctive features. He confined his attention to surface colonies in 18-24 hour cultures, grown on serum agar. These he described as circular, disc-like, and flattened, with regular contour. When viewed from above the colony surface appeared glassy, often with a slightly depressed center. When looked at from the side or by transmitted light they appeared as distinct milky rings enclosing a transparent center. This form he designated as the "ring type" of colony. Marked variations in colony size, which reached a maximum of 1.5-2 mm. in diameter, and in form were noted, including the round convex types (characteristic of young cultures) and the large, flat, mucoid forms. Other brief descriptions of the colonies of this organism may be found in most recognized text-books of bacteriology.

For general purposes, however, we have found Buerger's descriptions quite applicable to the majority of discrete colonies of type-specific pneumococci which appear in 24-48 hour cultures on neutral agar enriched with 15 per cent rabbit blood. We have also found the small, round, convex colonies (Fig. 1) to be more frequent in the early (16-24 hour) cultures, and the large, flat, and umbilicated or "ring forms," many of which show extraordinary variations in size, more characteristic of the 24-48 hour culture. The outline of the large forms is generally spherical or elliptical, the surface uniformly

smooth or mucoid (Figs. 2 and 3). One may perhaps make a further differentiation of the colonies formed by individual pneumococcus types for it has been recognized that Type III may produce larger and more mucoid colonies more constantly than the other types. However, these large, mucoid colonies do not seem to be distinctly characteristic for Type III and in the long run predictions as to the type of pneumococcus, based on the morphology of the colony, have proved more or less unreliable in our hands.

Rough Pneumococcus Colonies.

Brief descriptions of rough pneumococcus colonies have been made by Griffith (2), Yoshioka (3), Blake and Trask (4), Reimann (5), and Amoss (6). Reimann has characterized them as follows: "The . . . colony (R) appeared heaped up, thicker, more opaque, and less green than colonies of the other kind. Examined under the microscope the surface of these colonies was dull or finely granular. When pushed with a loop the colony seemed quite coherent and moved along as a whole. Occasionally the S colonies also had a granular surface but it was much less coarsely granular than that of the R colonies. Changes in color of the blood about the colonies due to methemoglobin formation were seen with both kinds of colonies."

Amoss has described the rough pneumococcus colonies as follows: "These were small, flat, and compact, greyish white in color, with an irregular surface. When picked with a platinum needle, the colonies were resistant and could be pushed about on the surface of the agar. With a platinum loop they could be removed *in toto*."

In general we have found the 24 hour colonies produced by the rough strains to be somewhat less variable in appearance than the smooth colonies of a similar age although again, different sizes and shapes are encountered in the 24 hour culture. As a rule the R colonies are smaller, generally circular, and there is greater predominance of elevated convex forms as opposed to the flat, disc-shaped or "ring forms" which are so characteristic of the S colonies (Fig. 4). In fact this tendency for central elevation rather than umbilication recalls the type of colony formed by certain strains of streptococci. In the 24 hour growth the surface is only slightly granular and the difference between the S forms at this stage is frequently not pronounced. After 48 hours, however, the roughening and granularity of the surface becomes more distinct (Fig. 5). At this stage the R

colony appears to be slightly more compact and opaque than the S, but in general the most prominent differences are those of size, shape, and texture of the surface. Subsequently these characteristics become even more pronounced and will be discussed later on in this paper.

The degree of methemoglobin formation about the R colonies may approximate that seen with the S. With some R strains, however, we have noted a tendency for the production of a small halo of hemolysis.

EXPERIMENTAL.

It is evident that typical surface colonies are only produced under optimum cultural conditions and their morphology may, of course, be influenced or altered by a host of incidental or extraneous factors, particularly in the case of an organism such as the pneumococcus which is so sensitive to its environment. Observations on the surface colonies produced by S and R strains of pneumococci under a small number of varied environmental conditions have therefore been included in this study, not only for the purpose of determining some of the conditions which promote or inhibit the formation of typical colonies, but also to familiarize ourselves with the different colony expressions which S and R strains may exhibit under varying conditions. The influence of the following factors has been observed: crowding, age, diminution of the blood in the culture media, and variation in the hydrogen ion concentration of the media. In the latter instances our observations have been limited to a study of the effects produced by relatively sudden changes in environment and we have not studied the colonies produced by organisms which have been grown repeatedly in altered media.

Crowding.—Our attention has been hitherto confined to the study of the discrete colony. It is evident, however, that with most bacteria the morphology of the growth upon an agar surface is greatly influenced by the heaviness of the inoculum and individual colony formation gives way to a confluent growth under conditions of moderate crowding. S pneumococci present no exception to this rule. When small numbers of individual colonies occur in sufficiently close contact so that their edges touch one another, they generally merge to form

confluent dumb-bell- or clover-leaf-shaped forms. With larger numbers and closer contact, a smooth surfaced growth may result in which all trace of individual colonies is lost except at the edges. If, however, the degree of crowding is extreme, large portions of the central areas of the growth may present quite a different appearance. Individual colonies give way to an irregular, amorphous, slightly elevated mass in which there may be myriads of tiny structures with irregular and roughened surfaces. One may note various zones in this process by observing the different aspects displayed in heavily seeded areas from the center out to the edge, where the typical colonies begin to appear (Fig. 6). In the irregular central mass sometimes one encounters irregular colonies with roughened surfaces which are comparable in appearance to those produced by true R pneumococci but under these conditions the presence of such colonies does not indicate that complete dissociation has taken place, for if transfers are made from such areas, typical smooth colonies are produced.

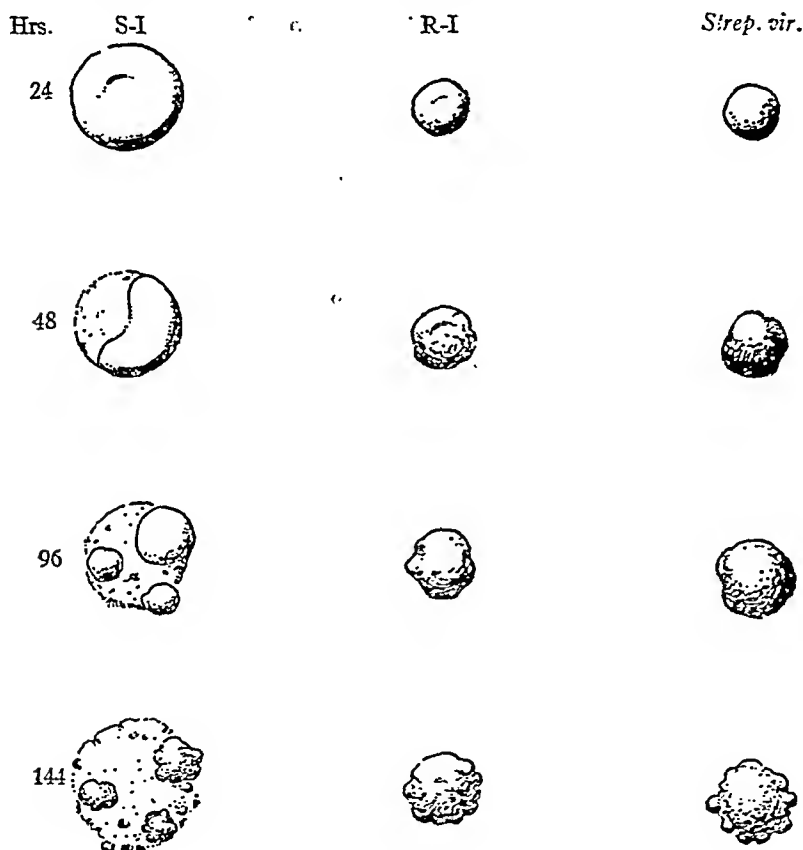
With R pneumococci the growth reaction to crowding is slightly different although the differences are relatively insignificant. The colonies tend to remain discrete to a degree which recalls the behavior of various types of streptococci. In heavily seeded areas individual colonies become reduced in size but still show less tendency to become confluent than does the S variety. With extreme crowding, however, confluence and amorphous growths occur, in which individual colony formation is lost, simulating the changes noted in the crowded S cultures.

Age.—It has long been known that striking morphological changes may be noted in the smooth pneumococcus colony if observed over the course of several days or a week. Such changes are by no means unique for the pneumococcus.

Hadley (1) has reviewed the extensive literature on this subject, emphasizing the phenomena of secondary and even tertiary colony formation which is common to a large number of bacterial species and may occur either with or without actual lysis of the primary colony.

Atkin (11) has recently emphasized some of the changes which the pneumococcus colony exhibits on serum agar plates of suitable reaction and of sufficient depth of media to allow growth to proceed for a considerable time, observing that the colony after increasing in size for 2 or 3 days subsequently became more or less completely autolyzed and transparent. On the succeeding day or two, evidences

of secondary colony formation were observed in which one, two, or more minute papillæ could be discerned on the surface of the original colony and these continued to grow for a week or two, eventually forming opaque secondary colonies which sometimes entirely covered the remains of the original site. He noted that these papillæ or secondary colonies did not undergo autolysis and that organisms from them were resistant to the lytic action of bile.



TEXT-FIG. 1.

In a series of daily observations upon *S. pneumoniae* colonies grown upon rabbit blood agar (pH 7.8) with sufficient depth of the media in the plate to retard drying for a period of a week, we have observed the changes which the colony exhibits during successive days and in particular the processes of colony autolysis and "papilla"

formation. The progressive changes exhibited by both S and R colonies, together with the similarity which the latter bear to the colonies produced by *Streptococcus viridans*, are illustrated in the diagrammatic sketch shown in Text-fig. 1.

As mentioned above, the typical "ring forms" are characteristic of the 24 hour S colony. At the end of 48 hours many of the colonies, having reached their maximum size, begin to show partial autolysis, which becomes progressive during subsequent days. The extent to which this process occurs differs with individual strains and is enhanced when the colonies are in close approximation to one another. At the 48 hour stage, however, frequently the central areas, comprising one-third or one-half of the original colony, appear to have "collapsed" while the remainder still retains its original characteristics, with rounded edges and a smooth surface. The collapsed or autolyzed portions are only slightly elevated from the surface of the media and present a granular or pitted surface, the line of demarcation between the two parts of the colony being generally quite sharp. After the 3rd day the majority of smooth pneumococcus colonies go on to complete autolysis without surviving papillæ and eventually appear under the microscope only as faint shadows (Fig. 7), although to the naked eye they are still visible upon the agar surface. On the other hand, by and after the 3rd day, signs of secondary growth may begin to appear in the form of papillæ, either in the surviving elevated portions or on the remainder of the colony site. These papillæ become more prominent on subsequent days. They are generally multiple and of variable size retaining at first a rounded contour and smooth surface (Fig. 8). With some strains there is a marked tendency to produce large numbers of papillæ so that the original colony site may be studded with dozens of tiny round, conical elevations (Fig. 9). Such papillæ seldom undergo lysis. They may steadily increase in size during the 4th, 5th, and 6th days, extending beyond the edges of the original colony site, thus producing in some instances secondary colonies, as large or even larger than the original. By the 6th day the surviving papillæ or secondary colonies are quite characteristic. If they are large at this stage their surfaces are frequently granular, often rugose, and dotted with protuberances. The edges are beaded with tiny nodules and the picture is not unlike that of an old R pneumo-

coccus or an old streptococcus colony rising from the remains of an autolyzed pneumococcus colony. Here again, although the secondary colony recalls that of an R pneumococcus and, as Atkin has shown, the actual organisms from such a colony do exhibit variant properties, it is evident that complete dissociation into a new strain has not taken place, for if these rough secondary colonies are transferred to fresh media, typical smooth colonies result.

With true R pneumococci the daily changes exhibited by the colonies over a period of a week differ materially from those of the S variety. The 24 hour R colony has already been described. In 48 hour cultures it is larger; it may be either rounded or flat and occasionally shows a slight central depression. Instead of undergoing autolysis as S colonies do, it continues to grow and the characteristics of roughness, opacity, and compactness become emphasized with increasing age, again recalling the changes exhibited by *Streptococcus viridans* more than those of the S pneumococcus. The colony may continue to increase steadily in size, tending to flatten out during the 3rd, 4th, and 5th days often by spreading out from the base either in the form of a scalloped or beaded edge. Tiny nodules or papillæ appear on the surface or edge of the colony about the 4th day, recalling the appearance of those noted in the S colonies.

Diminution of Blood in the Culture Media.—There are undoubtedly a number of conditions under which atypical pneumococcus colonies may be produced upon media which are deficient in suitable nutritive requirements for luxuriant growth. Our studies have been limited to a series of observations on the effect produced by diminishing the amount of rabbit blood added to nutrient agar.

If the concentration of blood is reduced to a point where the pneumococcus begins to grow poorly, distinctly atypical colonies occur. This phenomenon generally occurs for S types when the concentration of rabbit blood added to nutrient agar falls below 5 per cent. The colonies under these conditions appear small and rough. Here again the organisms from the colonies cannot be designated as true R forms for on transfer to suitable media they produce smooth colonies and may be shown to be type-specific by appropriate agglutination tests.

Acid and Alkaline Media.—Finally the effect which variations in the hydrogen ion concentration of the media exert upon colony formation of both S and R pneumococci has been studied.

The experiment was performed in the following manner: Twelve lots of nutrient agar were prepared covering a range in pH of 6.2-9.4, and to each lot 15 per cent rabbit blood was added. Owing to technical difficulties the final pH of the blood agar was not estimated and we have consequently designated the samples of media at which growth could be initiated on the acid and alkaline side as about pH 7.0 and about pH 8.3 respectively. Daily observations and drawings of the colonies were procured covering the changes exhibited over a period of a week by each strain on the different lots of media.

TABLE I.
Differential Properties of S and R Pneumococcus Colonies.

Property	S	R
Size	Marked variability, ranging from 0.1-2 mm. in diameter. Original colonies reach maximum size between 24 and 48 hrs.	Moderate variability, ranging from 0.1-1 mm. in diameter. Colonies may show progressive increase in size over a period of 5-7 days
Shape	Round or elliptical, often exhibiting a central depression. Tend to be confluent	Round, elliptical, and often irregular. Tend to be discrete
Surface	Smooth during first 48 hrs. with subsequent irregularity accompanying autolysis	Becomes progressively rough after 24 hrs.
Autolysis	Marked in 36-96 hr. cultures	Practically absent during 1st wk.
Secondary colony formation	Marked in 36-96 hr. cultures	Present in 48-96 hr. cultures
Methemoglobin formation	Present	Present, but may be replaced by slight hemolysis

From this study we have made the following general observations. On the extreme alkaline side (pH about 8.3) the colonies of S strains of pneumococci proved to be quite atypical, small, and rough, resembling somewhat the colonies produced by these same organisms when grown upon media poor in blood. Here again, however, the organisms could not be classified as true R pneumococci for they showed colony autolysis, and if transferred to more suitable media, immediately produced typical smooth colonies. On the acid side the

S pneumococcus colonies showed little deviation from the normal picture with the possible exception of the fact that with certain strains of Type III the colonies occasionally underwent more rapid lysis coupled with early and extensive formation of secondary colonies, a phenomenon which has been noted by Atkin (11). An interesting feature, however, was that with one of the Type III strains typical, rough colonies appeared on the 2nd or 3rd day, scattered among the smooth whenever it was plated upon acid media. The organisms from these colonies proved to be true R pneumococci.

With R pneumococci, the colonies proved quite constant in appearance when grown in media covering a wide range of hydrogen ion concentrations, and apart from the fact that they tended to be somewhat smaller in strongly acid and alkaline media we have failed to note other changes.

General Characteristics of Smooth and Rough Colonies.—Some of the properties which have been found characteristic for S and R pneumococcus colonies have been assembled, for the sake of brevity, in tabular form (Table I). We have only mentioned those which are of more value in differentiating the two groups.

DISCUSSION.

The general appearances and properties of typical S and R pneumococcus colonies have been briefly reviewed with a discussion of the distinctive colony characteristics presented by each group. Emphasis has been laid upon the more labile nature of the S colony which is characterized by the rapid growth of a smooth surfaced disc-shaped structure which subsequently undergoes rapid autolysis and secondary colony formation. The R colony is characterized by a rough surface, gradual and progressive increase in size over a period of several days, failure to undergo rapid autolysis in early cultures, and more limited secondary colony formation, recalling in some measure the appearance and behavior of the colonies produced by members of the *Streptococcus viridans* group.

It has also been shown that mere roughness of the surface of a pneumococcus colony does not necessarily indicate that the organisms from such a colony are true R forms for some of the observations given above show that S pneumococci may, under a variety of different

environmental conditions, give rise to "pseudo rough" colonies. The distinction between "pseudo rough" and true R colonies is that organisms from the former retain their type specificity and immediately give rise to typical S colonies when transferred to suitable environment, whereas, it is recognized that the organisms from true R colonies do not revert to S forms with such apparent ease. It is conceivable, however, that the organisms in "pseudo rough" colonies may be closely related to some of the intermediate forms which have been described.

SUMMARY.

The characteristic appearances exhibited by the surface colonies of both S and R pneumococci in 24 and 48 hour cultures upon rabbit blood agar have been reviewed. Emphasis has been laid upon the behavior and structure of the colonies formed by R pneumococci, their frequent similarity to the colonies formed by certain strains of *Streptococcus viridans*, and their failure to undergo rapid autolysis in the first 48-96 hours, a phenomenon which is highly characteristic of the S pneumococcus colonies. With the S pneumococci it has been shown that "pseudo rough" colonies may be immediately produced under certain unfavorable cultural conditions but such changes in colony morphology as these do not indicate that complete dissociation has taken place and that the organisms may be classified as true R pneumococci.

In conclusion I wish to express my thanks to Miss Margaret McClintock for her assistance in the technical work of this study.

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EXPLANATION OF PLATES.

PLATE 31.

FIG. 1. Colonies formed by *S. pneumococci* (Type II) in an 18 hour culture on rabbit blood agar. Some of the small convex forms show beginning umbilication. \times about 20.

FIG. 2. Colonies formed by *S. pneumococci* (Type II) in 24 hour culture, showing large, flat, disc and ring forms. \times about 40.

FIG. 3. Colonies formed by *S. pneumococci* (Type III) in 24 hour culture showing rounded and elliptical forms. Typical central umbilication is noted in the middle colony. \times about 50.

FIG. 4. Colonies formed by *R. pneumococci* (R-I) in 24 hour culture. Most of them assume a convex form. The tendency for central elevation rather than umbilication is emphasized by the dark shadows surrounding each colony. The surface is definitely granular. \times about 50.

FIG. 5. Colonies formed by *R. pneumococci* (R-II) in a 48 hour culture. Roughening and granularity of the surface are more pronounced at this stage. \times about 50.

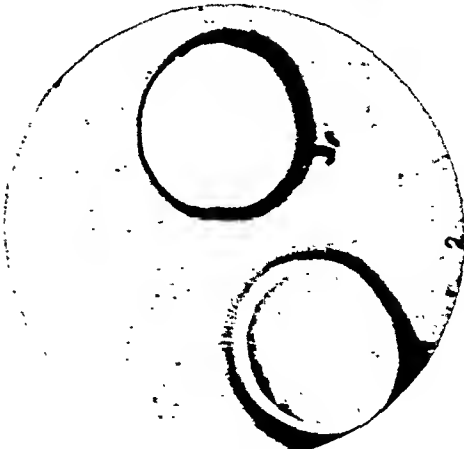
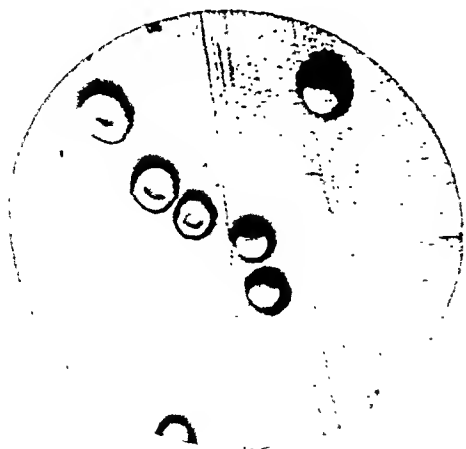
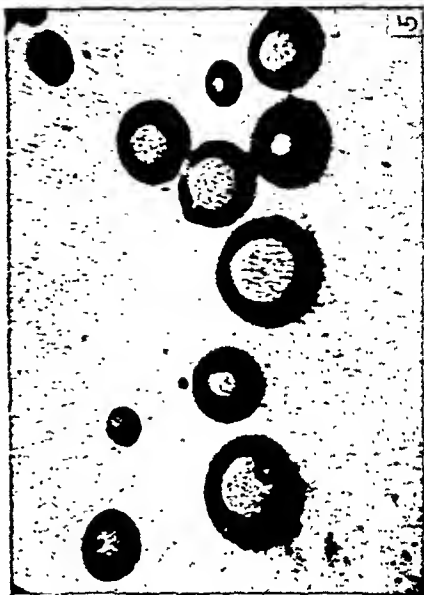
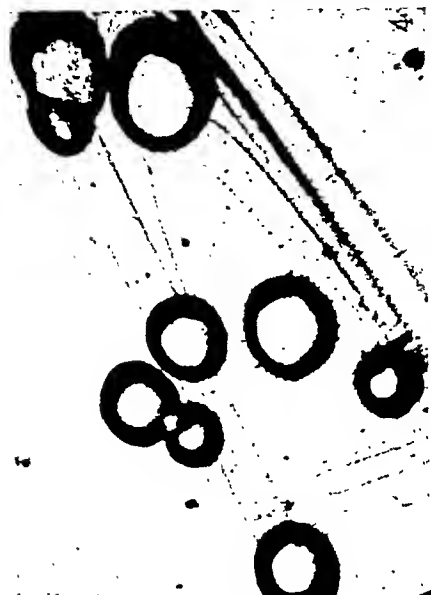
PLATE 32.

FIG. 6. Showing the effect of crowding as exhibited by a 24 hour culture of *S. (Type II) pneumococci*. A few typical colonies survive at the edge of the growth. The central areas present an irregular amorphous appearance. \times about 20.

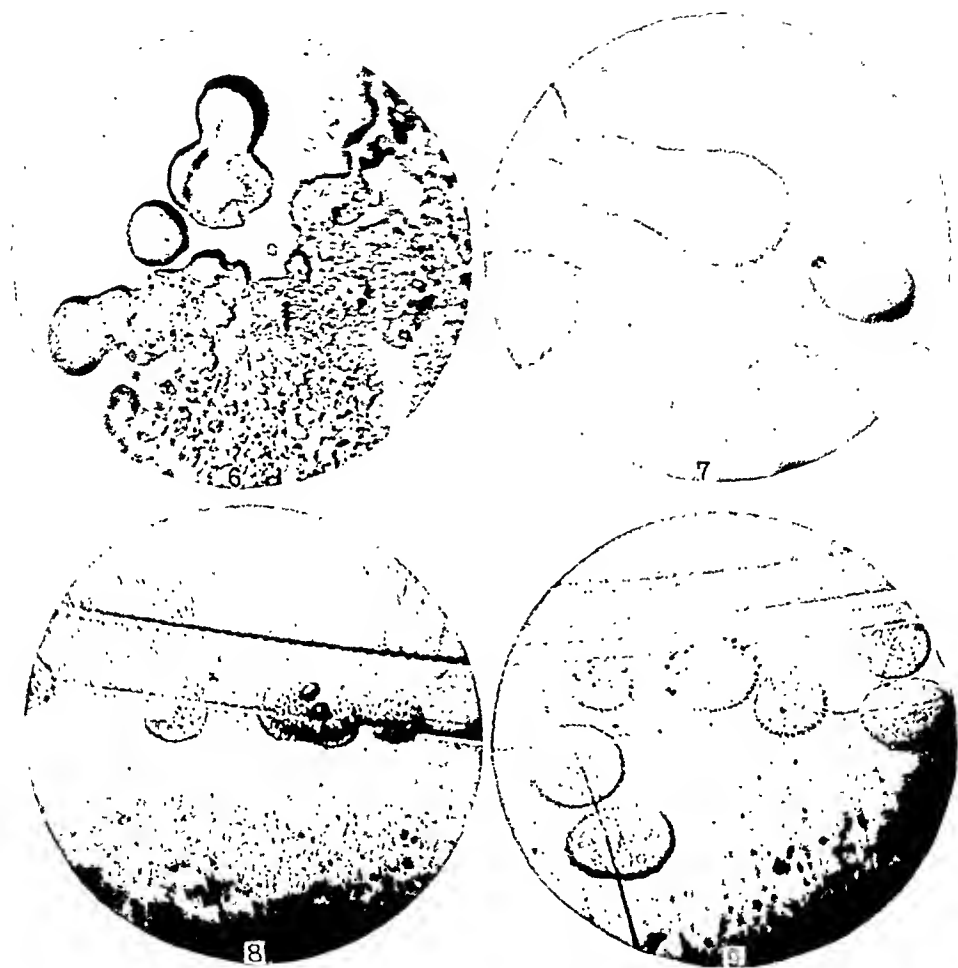
FIG. 7. Colonies of *S. pneumococci* (Type III) in a 72 hour culture. Moderate lysis has taken place; cf. Fig. 3. \times about 40.

FIG. 8. Colonies of *S. pneumococci* (Type I) in a 72 hour culture showing partial lysis with surviving papillae on one of the colony sites. \times about 30.

FIG. 9. Colonies of *S. pneumococci* (Type III) in a 72 hour culture showing partial lysis and multiple papillae about the edges of individual colony sites. \times about 30.



(Paul: Smooth and rough pneumococcus colonies.)



(Paul: Smooth and rough pneumococcus colonies.)

THE OCCURRENCE OF ROUGH PNEUMOCOCCI IN VIVO.

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INTRODUCTION.

The establishment of recognized groups of pneumococcus variants such as the rough or R and possibly intermediate forms has raised the question as to whether these variants, particularly the R forms, play any rôle in the course of an active pneumococcus infection or in the epidemiology of such infections. Up to the present time most of the studies upon R pneumococci have been made upon those forms isolated *in vitro* as a result of subjecting smooth or S forms to various cultural or environmental conditions. Our knowledge of their pathogenicity or relationship to the human host is as yet limited, but recent work with animals has suggested that the isolation of these variants of reduced virulence during the course of pneumococcus infection may be of significance in our general conceptions of the infectivity of the disease and the host response.

Wadsworth and Sickles (1) have shown that the pneumococcus multiplying in the tissues of the immunized animal becomes attenuated, and they have reported the isolation of several strains of these attenuated pneumococci from the blood stream and heart valves at autopsy, of horses which had been immunized with Type I pneumococci. These strains showed a loss, in varying degree, in virulence, capacity of capsule formation, susceptibility to phagocytosis, and type specificity. The antigenic activity as an immunizing agent and the production of soluble specific substance were also altered. With some of these strains it was found that the typical pneumococcus characteristics could be restored by one or two passages through a mouse, while others remained atypical.

In a study on the pathogenicity of degraded pneumococci Reimann (2) has discussed the importance of determining whether the R variants ever appear *in vivo* and, if so, under what conditions. He states that: "Although they have been carefully searched for, R forms have not been encountered in the cultures of sputum or blood of patients, either during the period of pneumococcus infection or during convalescence, or in direct cultures from the lungs at autopsy." On

the other hand, this author has shown that under experimental conditions a degradation from the S to the R form actually does take place in cultures of pneumococcus growing in agar subcutaneously embedded in guinea pigs and in agar enclosed in vials subcutaneously embedded in rabbits. However, this change was never complete and when the intermediate or R forms did appear they were always accompanied and usually exceeded in number by the S forms. Another interesting observation was that pure cultures of the avirulent R forms remained viable in subcutaneous foci for weeks.

Previously there have been a number of reports on the isolation of atypical pneumococci from lesions in animals and man. In 1907 Buerger and Ryttenberg (3), in studying the morphology and cultural characteristics of pneumococci in human exudates and human blood, concluded that wide variations from the typical forms may occur. These atypical forms could be reverted by animal inoculation soon after their isolation, but 2 months after isolation the organisms had acquired fixed cultural characteristics and repeated animal inoculation failed to bring about a change.

Later Rosenow (4, 5), in a series of studies on pneumococci isolated from cases of endocarditis, described these organisms as "modified" pneumococci. They were atypical from the standpoint of their morphology, methemoglobin formation, inulin fermentation, and the fact that the colonies produced by these organisms on blood agar adhered more closely to the surface of the culture medium than those produced by typical pneumococci. However, by cultivating these strains in normal serum or blood and by animal inoculation a reversion to typical pneumococci was established.

Bull (6) has also reported that certain changes may be undergone by pneumococci during the course of experimental septicemia in dogs. He noted that pneumococci isolated as the infection was subsiding were more susceptible to the action of immune serum than the original cultures which had been injected. In one fatal case the pneumococci isolated on the 9th day grew in chains and were avirulent.

From the observations quoted above it would seem likely that in the earlier work some of the avirulent variants which have been isolated by Rosenow (5) and others from human patients may have been related to the R or intermediate pneumococcus forms. The more recent observations of Wadsworth and Sickles (1) and Reimann (2), however, have shown conclusively that such degradation of pneumococci may be produced experimentally *in vivo*.

In order to study this question we have limited ourselves to a single aspect of the major problem in an effort to determine whether or not one may isolate proved R pneumococci from the human body and, if so, under what conditions. The observations given below present

evidence which we believe answers the first question in the affirmative, but the data which deal with the second question are as yet too limited to lead to a profitable discussion.

The main feature of our work has consisted in a differential study of a group of organisms which we have isolated in a small number of instances from the sputum of individuals suffering from infections of the respiratory tract. The strains which we have studied have been primarily selected on the basis of colony differentiation together with their ability to produce methemoglobin when grown upon the surface of rabbit blood agar and the study has essentially resolved itself into the differentiation of these forms from strains of *S. pneumoniae* or from strains of *Streptococcus viridans*,* and possibly other species of allied bacteria.

Methods.

For colony differentiation 18-24 hour cultures were made on fresh rabbit blood agar plates (pH 7.8) and studied according to the methods outlined in a previous paper (9).

Broth cultures were made in 1 per cent dextrose broth to which rabbit blood had been added in a concentration of 10 per cent.

Two standard *R* strains of pneumococci, *R*-I and *R*-II, were employed as controls throughout the work. These were obtained from The Rockefeller Institute for Medical Research through the kindness of Dr. O. T. Avery. The *S* strains of type-specific pneumococci, also used as controls, were obtained from the sputum of cases of lobar pneumonia which occurred at the Pennsylvania Hospital during the winter of 1926-27, all of which had been subjected to one animal passage. Five strains of *Streptococcus viridans* and one strain of hemolytic streptococcus, also used for controls, were obtained from recent blood cultures of cases of subacute bacterial endocarditis and hemolytic streptococcus septicemia respectively. All the control strains were kept at room temperature on rabbit blood agar with weekly transfers.

The anti-*S* or type-specific antipneumococcus serum employed for precipitation

* It is evident that any investigation which attempts to differentiate the organisms of the so called green-producing streptococcus group and *R. pneumoniae* should include a discussion of the possible relationship between these two forms. Morgenroth and his collaborators (7) have recently reported that by special methods it was possible to transform pneumococci into streptococci with the production of various intermediate forms. The evidence to support such a view is not, however, convincing, and in a critical review of this work Reimann (8) has been unable to arrive at similar conclusions.

and agglutination tests was obtained from the Division of Laboratories and Research, New York State Department of Health.

EXPERIMENTAL.

During the past 18 months we have been engaged in a series of pneumococcus colony studies on cultures which have been obtained from the spinal fluid, mastoid, chest cavity, and other regions of the body

TABLE I.

Type of Case and Stage of the Disease from Which the Twelve Strains (X-1 to 12) Were Obtained.

No.	Initials	Age yrs.	Sex	Race	Pulmonary lesion	Stage of disease
1	J. D.	50	F.	W.	Bronchopneumonia	Early in active stage
2	A. B.	7	"	"	Mild postoperative bronchopneumonia	" " " "
*3	J. M.	39	M.	"	Lobar pneumonia (Type II)	" " " "
*4	" "	39	"	"	" "	" " " "
*5	A. S.	28	"	"	" "	" " " "
*6	" "	28	"	"	" "	" " " "
*7	M. S.	37	F.	C.	Lobar pneumonia (Type IV) (fatal case)	Late " " "
*8	" "	37	"	"	" "	" " " "
9	T. S.	28	"	W.	Bronchopneumonia	" " " "
*10	J. M.	39	M.	"	Lobar pneumonia (Type II)	Early convalescence
11	R. S.	42	"	"	Bronchopneumonia. Active tuberculosis	During active stage of bronchopneumonia
12	I. A.	26	"	C.	Lobar pneumonia (Type I)	Late in active stage

* Nos. 3, 4, and 10 represent the same patient, as do also Nos. 5 and 6, 7 and 8.

taken during the active stage of a pneumococcus infection. In none of these cultures, many of which had been obtained from suppurative lesions of varying age, were we able to detect the presence of R pneumococci.

R Pneumococci in Sputum Cultures.

In attempting to isolate R pneumococci from sputum our attention was first directed to a survey of the colonies which are formed by

different strains of *Streptococcus viridans* in sputum cultures from normal individuals and from individuals representing a wide variety of clinical conditions. The *viridans* colonies which occur in sputum cultures apparently exhibit a very wide degree of variation. Many of them prove to be quite different from those of R pneumococci while others are practically indistinguishable. From the latter groups a number of strains (about 40) were isolated from suspected colonies and from these twelve bile-soluble strains were selected for further study. These strains have been designated X-1 to 12 inclusive, and are listed in Table I together with the diagnoses of the cases from which they were obtained. The data presented in this table merely show the type of cases we have studied. No conclusions regarding the degree of incidence of R pneumococcus in the sputum, either in cases of this type, or in normal individuals, have been drawn from such a limited number of observations.

Probably one of the most satisfactory methods for establishing the identity of a variant organism is to effect a reversion to a more easily recognizable form and subsequently to identify the resulting organism. We have been unable to take advantage of this method in these studies, however, for to date we have been unsuccessful in producing a reversion from R to S forms either with the control strains of R pneumococci or with the suspected strains of R pneumococci which have been obtained from the sputum cultures. Consequently other methods have been employed for the identification of the suspected strains. Assuming *a priori* that the suspected organisms might be classified either with the large group of so called *Streptococcus viridans*, or as true R pneumococci, we have used three methods, hitherto considered of value in differentiating rough pneumococci from streptococci, namely, those of determining (a) the degree to which the organisms are soluble in bile, (b) the degree to which they undergo autolysis in saline solution, and (c) their agglutination reactions with anti-R pneumococcus serum.

(a) *Bile Solubility*.—Although the lytic action of ox bile and sodium taurocholate solutions is widely employed as a useful method for differentiating pneumococci from *Streptococcus viridans*, it is recognized that some strains of pneumococci are more resistant to the action of bile than others. Reimann (8) has noted that R pneumococci are more

resistant to the lytic action of bile than *S* pneumococci. Occasionally one may encounter strains of *S* pneumococci which are similarly resistant and to emphasize this fact we have included such a strain among the *S* controls. We are not aware, however, that any of the recognized strains of *Streptococcus viridans* possess the property of being dissolved in ox bile or in the lower concentrations of sodium taurocholate solutions used in the following experiments.

TABLE II.

Viability of Strains after Exposure to Bile and Sodium Taurocholate Solutions.

Strain	Ox bile	Per cent of added sodium taurocholate solutions				Strain	Ox bile	Per cent of added sodium taurocholate solutions	
		2.5	5	10	20			2.5	5
S-I (1)	0	0	0			X-1	0	0	0
S-II (1)	++	0	0			X-2	0	0	0
S-II (2)	+	+	+	+	0	X-3	0	0	0
S-II (3)	0	0	0	0		X-4	0	0	0
S-III (1)	0	0	0			X-5	0	0	0
S-III (2)	0	0	0			X-6	0	0	0
S-III (3)	0	0	0			X-7	0	0	0
R-I	0	0	0			X-8	0	0	0
R-II	+	0	0			X-9	0	0	0
<i>Strep. vir.</i> (1)	+++	+++	+++	++	++	X-10	0	0	0
" " (2)	+++	+++	+++	++	++	X-11	0	0	0
						X-12	0	0	0

+++ = profuse growth.

++ = moderate growth.

+ = very few colonies.

0 = no growth.

Methods of Testing Bile Solubility.—The organisms from a 24 hour broth culture were taken up in a corresponding volume of salt solution. Equal parts of ox bile and solutions of sodium taurocholate in concentrations of 2.5, 5, 10, and 20 per cent were added to these suspensions and they were placed in the incubator for 2 hours at 37°. By culturing a loopful of suspension on rabbit blood agar, both at the beginning and at the end of the experiment, the viability of the organism after exposure to bile was determined.

Results.—The results are given in Table II. Some of the *S* strains showed evidence of being more or less resistant to the lytic action of

TABLE III.
Viability of Strains after Exposure to Saline Solution.

Strain	Hrs. of incubation in saline solution					Strain	Hrs. of incubation in saline solution				
	0	2	4	6	18		0	2	4	6	18
S-I (1)	+	0	0	0	0	X-1	++	++	++	++	++
S-I (2)	+	0	0	0	0	X-2	+	++	++	+	+
S-I (3)					0	X-3	+	0	0	0	0
S-II (1)	++	++	+	0	0	X-4	++	+	+	+	0
S-II (2)	++	++	+	0	0	X-5	++	++	++	+	0
S-II (3)	++	0	0	0	0	X-6	++	++	++	+	0
S-III (1)	++	++	++	++	0	X-7	++	++	++	++	0
S-III (2)	++	++	0	0	0	X-8	++	++	++	++	0
S-III (3)	++	++	++	++	0	X-9	++	++	++	++	0
R-I	++	++	++	++	0	X-10	++	++	++	++	0
R-II	++	0	0	0	0	X-11	++	++	++	++	0
<i>Sreph. vir.</i> (2)	++	++	++	++	++	X-12	++	++	++	++	++
" " (3)	++	++	++	++	++		++	++	++	++	++
" " (5)	++	++	++	++	++		++	++	++	++	++

+++ = profuse growth.

++ = moderate growth.

+ = few colonies.

0 = no growth.

bile and sodium taurocholate solutions, but all were dissolved in the concentrations employed, although the strains of *Streptococcus viridans* survived in these same concentrations. One of the two control R strains proved to be slightly resistant to ox bile. About one-third of the colonies chosen from sputum cultures on the basis of their resemblance to the colonies of R pneumococci proved to be bile-soluble according to this method. These have been designated as X-1 to 12 and are shown in the right hand columns of the table.

(b) *Autolysis in Saline Solution*.—The readiness with which pneumococci, particularly the R forms, undergo autolysis when suspended in saline solution at 37°, as opposed to streptococci of all varieties which undergo autolysis very slowly, has also been emphasized by Reimann (8). We have used this method in an attempt to differentiate the two organisms. The results are given in Table III.

Methods.—The organisms from a 24 hour broth culture were taken up in a corresponding volume of physiological salt solution and placed in the incubator at 37°C. for a period of 18 hours. Cultures were made from these suspensions at stated intervals.

Results.—It will be noted that with all the control S and R strains of pneumococci the organisms had ceased to be viable at the end of 18 hours, whereas the *Streptococcus viridans* strains grew as readily at the end of 18 hours exposure to salt solution as at the beginning. Three of the X strains grew at the end of 18 hours while all the others underwent autolysis.

(c) *Immunological Reactions*.—Lancefield (10) has shown that the protein of various strains of *Streptococcus viridans* is immunologically identical with that of the pneumococcus. Consequently serum produced by each *Streptococcus viridans* agglutinates all R pneumococci. On the other hand individual strains of *Streptococcus viridans* seem to possess a substance comparable to the soluble specific substance of the pneumococcus which masks or prevents agglutination of *Streptococcus viridans* by anti-R pneumococcus serum and in testing six strains of streptococcus with anti-R pneumococcus serum Reimann (8) was unable to get any evidence of agglutination. It is recognized, however, that anti-R pneumococcus serum will agglutinate and precipitate the protein from all R pneumococci, regardless of their original type (11).

In the light of these observations the theoretical question arises as to whether degraded forms of the streptococcus group which have lost the power of elaborating their soluble specific substance may not occur *in vivo*. If such is the case one might expect that "degraded streptococci" would be agglutinated by anti-R pneumococcus serum. Our experimental data have been reviewed critically in this light and we

TABLE IV.
Agglutinating Reactions with Anti-R Pneumococcus Serum.

Strain	Dilution of anti-R pneumococcus serum			Strain	Dilution of anti-R pneumococcus serum		
	1/10	1/20	1/40		1/10	1/20	1/40
S-I (1)	+++	++	±	X-1	0	0	0
S-I (2)	++	+	±	X-2	0	0	0
S-I (3)	+++	++	+	X-3	++	++	+
S-II (1)	+	±	0	X-4	0	0	0
S-II (2)	0	±	0	X-5	++	++	+
S-II (3)	0	0	0	X-6	0	0	0
S-III (1)	+	+	±	X-7	0	0	0
S-III (2)	0	0	0	X-8	0	0	0
S-III (3)	+	±	0	X-9	+	0	0
R-I	++++	+++	++	X-10	0	0	0
R-II	+++	+++	+++	X-11	±	0	0
<i>Strep. vir.</i> (2)	0	0	0	X-12	±	0	0
" " (3)	0	0	0				
" <i>hzm.</i>	0	0	0				

++++ = firm disc.

+++ = disc easily broken up.

++ = coarse agglutination.

+

± = faint agglutination.

0 = no agglutination.

have consequently not accepted as fact that any methemoglobin-producing diplococcus which is agglutinated in appropriate dilutions by anti-R pneumococcus serum is necessarily an R pneumococcus. We have assumed, however, that such agglutination reactions are of relative importance if taken in conjunction with other findings.

Methods.—The anti-R pneumococcus serum was prepared according to the principles of Cole and Moore (12). Bacteria from 18 hour broth cultures of two

strains of R pneumococci, R-I and R-II, were suspended in salt solution and killed at 56°C. Rabbits were inoculated with this suspension, a preliminary series of subcutaneous injections being followed by two series of six intravenous injections each.

The agglutinating reactions were run in duplicate upon saline suspensions of washed bacteria. Readings were made after incubation at 37°C. for 5 hours and again after 12 hours in the ice box.

Results.—The results are shown in Table IV. It will be noted that several of the S strains gave agglutination reactions in moderately high dilutions of the anti-R serum. These results are not altogether in accord with those of Reimann (11) but the degree of dissociation which may have occurred in these strains when suspended in saline solution has been an uncontrolled variable in our hands. No agglutination was obtained with the three control strains of streptococcus.

TABLE V.
Reactions Exhibited by Suspected R Pneumococcus Strains.

Strain.....	1	2	3	4	5	6	7	8	9	10	11	12
Bile solubility.....	+	+	+	+	+	+	+	+	+	+	+	+
Saline autolysis.....	0	0	+	+	+	+	+	+	+	+	+	0
Agglutination.....	0	0	+	0	+	0	0	0	0	0	0	0

+ = reaction typical for R pneumococci.

0 = reaction atypical for R pneumococci.

Of the X strains only two gave agglutinating reactions which are comparable to those of true R pneumococci.

A summary of the findings exhibited by these organisms is given in Table V.

Of the twelve strains of suspected R pneumococci obtained from sputum cultures only two consistently exhibit reactions which are typical for R pneumococci. No attempt has been made to explain the atypical reactions nor to classify the ten strains which have exhibited such reactions. We do not feel, however, that we have as yet evidence to say that any of these ten strains may be considered as true R pneumococci although it is not unlikely that they may be intermediate or related forms. We have felt, on the other hand, that the two

remaining strains may be justifiably classified, on the basis of the three reactions given above, as R pneumococci.

The evidence suggests that R pneumococci may be demonstrated in the sputum of patients suffering from pneumonia but these forms do not seem to be very common if we consider that on the basis of colony differentiation about forty strains were originally selected and but two of these were identified as true R pneumococci.

SUMMARY.

In a survey of about forty rough methemoglobin-producing colonies in sputum cultures from a series of individuals suffering from respiratory infections, twelve bile-soluble strains of suspected R pneumococci have been isolated for study. Two of these twelve strains have shown both autolysis in saline solution and serological reactions characteristic of R pneumococci.

The findings offer evidence that R pneumococci may occasionally occur in human sputum. Their significance as regards the epidemiology of pneumococcus infections and of host response is alluded to.

In conclusion the author wishes to express his appreciation to Miss Margaret McClintock for her assistance in the technical work of this study.

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A STUDY OF THE HERING-BREUER REFLEX.

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INTRODUCTION.

The observation has been made by several investigators that a rapid respiratory rate depends on intact vagal conduction. In experiments on anesthetized rabbits, Scott (1908) showed that an increase of carbon dioxide (not above 6 to 7 per cent) in the inspired air after the vagi had been divided was followed by an increase in the depth of breathing, but that the rate remained practically unaltered. On the contrary, the response of animals with intact vagi was an increase in rate, as well as in depth of respiration. Porter and Newburgh (1917) found that the rapid respirations occurring in dogs in whom they had produced an experimental pneumonia became normal after cocainizing the vagus nerves. They had noted before this that if the vagal trunks were sectioned previous to infecting the animals the respirations did not become rapid (1916). Dunn (1920), and still later Binger and his coworkers (1924), demonstrated that the rapid and shallow breathing, produced experimentally in animals by the intravenous injection of a suspension of potato starch granules, disappears immediately if the vagus nerves are divided or frozen. It was shown later that animals in whom the vagi had been sectioned several months previous to the injection of starch granules did not accelerate their rate (Binger and Moore, 1926). It would appear from the investigations of these latter authors (Dunn and Binger) that the Hering-Breuer reflex was enormously exaggerated under the conditions of their experiments. Presumably section of the vagi blocked the peripheral stimuli that were essential to the rapid rate.

In spite of the many studies that have been made of the vagal influence on breathing, the manner in which the vagal nerve endings are excited is not yet clear. Our interest in this subject developed from

an effort to explain the rapid and shallow breathing seen clinically in pneumonia, and produced experimentally in animals by the intravenous injection of potato starch granules. The experiments that will be presented were designed to study the nature of the Hering-Breuer stimulus.

Historical.

Early last century Legallois (1812) observed that section of the vagus nerves is followed by slowing and deepening of the respirations.

Hering and Breuer (1868), in a series of experiments performed on dogs and rabbits, both with and without anesthesia, showed that inflation of the lungs at once produces a movement of expiration and deflation a movement of inspiration. These observations were made both when the animals breathed naturally and when the lungs were expanded or collapsed artificially. The influence was not present when the vagi were divided. On the basis of their evidence Hering and Breuer formulated a theory for the self-regulation of respiration. They concluded that the respiratory center was continuously influenced through the vagi by the movements of the lungs, expansion checking inspiration and initiating expiration, and collapse checking expiration and initiating inspiration. Hering and Breuer also described a pause in respiratory movement following distention of the lungs. They explained this as an inspiratory inhibition due to vagal excitation following expansion.

The observations of Hering and Breuer were confirmed and elaborated by Head (1889), and later by Christiansen and Haldane (1914), and Haldane and Mavrogordato (1916). Head showed that inflation of the lungs produces an instant and complete relaxation of the diaphragm, and that if air be sucked out of the lungs the diaphragm will go into a state of tonic contraction. Both of these effects were absent if the vagi had been previously cut.

Christiansen and Haldane were the first to study the effect of distending the lungs on the human respiration. Distention was provided at the end of expiration by having the subjects breathe from a bag containing air, and so weighted that the air was under a pressure of 6 to 8 cm. of water. The respirations were recorded with a stethograph. When the lungs were distended in this manner, the respiratory movements invariably ceased, for usually about half a minute.¹ That the pause was not due merely to lowering of the alveolar CO_2 pressure was shown by the fact that it was still produced when the air in the bag contained 7.3 per cent of CO_2 and 8.2 per cent of O_2 . At this time, Christiansen and Haldane explained the pause that they had observed in man as an Hering-Breuer "inhibitory" effect.

¹ The pause in respiratory movements referred to here is the primary pause and must be distinguished from a secondary pause that was also observed. This latter was clearly shown to be related to a lowered CO_2 percentage in the inspired air and was described as a true chemical apnea.

This interpretation, however, was subsequently changed by Haldane (1922), and the pause has since been described as simply a prolonged expiratory effort.

Haldane and Mavrogordato demonstrated in man that interruption of either respiratory phase interrupts the previous rhythm of the respiratory center. If expiration is interrupted, there follows a prolonged expiratory phase during which the intrapulmonary pressure gradually rises. This is finally terminated by an inspiratory effort. If inspiration is obstructed, a prolonged inspiratory effort follows, but under these conditions the intrapulmonary pressure falls and the negative phase is terminated by an expiratory effort. Commenting upon these and the foregoing experiments, Haldane (1922) says: "The respiratory center does not act independently of the lung movements, but inspiratory or expiratory discharge of the center goes hand in hand with actual inspiration or expiration, as if the center were one piece with the lungs."

Boothby and Berry (1915) also studied the effects of distention of the lungs on the respiratory rhythm in man, and extended their observations to normal and vagotomized dogs. The lungs were distended by having the subjects breathe from a spirometer so weighted that the air was under a pressure of 8 to 16 cm. of water. The dogs did not require anesthesia. The respiratory movements were recorded qualitatively by means of a pneumograph, and quantitative curves of the "coordinated total respiratory movements" were written by the spirometer. None of the curves that they obtained showed a primary inspiratory inhibition. In regard to the experiments of Hering and Breuer, they say: "The pause noted by Hering and Breuer is not at the top of inspiration but at the bottom of expiration, that is, the next expiratory phase is prolonged and inspiration is delayed, or, as they say, 'inhibited,' by vagal stimulation." In experiments on dogs, in which the pulmonary branches of both vagi had been divided, Boothby and Berry obtained several instances of a short apnea on distention of the lungs. They concluded that the vagi do not transmit impulses which, according to Hering and Breuer, arise from distention of the lungs and inhibit inspiration.

Einthoven (1908) studied the action currents in the peripheral end of the divided vagus by means of a string galvanometer. Anesthetized dogs were used and the vagus was divided high up in the neck. Records of the electrical changes in the nerve were made by photographing the image of the galvanometer string. When the animals breathed naturally curves were obtained that showed undulations synchronous with the respiratory movements. Additional experiments were carried out on the effects of insufflating and deflating the lungs. These showed that the excitatory state of the vagus nerve endings was related to lung volume and not to intrapulmonary pressure. In other experiments of this type Einthoven found that electrical changes occurred in the peripheral end of the cut nerve that were synchronous with artificial deflation of the lungs. He accepted his findings as evidence of the presence of two kinds of pulmonary vagus fibres, and concluded that his experiments gave support to the theory on the self-regulation of respiratory movements that had been advanced by Hering and Breuer.

Schafer (1919) admits that respirations are affected by influences coming from the lungs, but calls attention to the fact that double vagotomy is not always followed by constant effects. He believes that the results of vagotomy may be influenced considerably by obstruction at the glottis from paralysis of the laryngeal muscles and a falling together of the thyroarytenoid ligaments. When this is avoided Schafer states that the slowing of breathing is often absent or transitory, and that animals may survive section of the vagi indefinitely.

Pi Suñer and Bellido (1921) claim to have produced evidence to show that the vagal nerve endings in the lungs are susceptible of being stimulated by high concentrations of carbon dioxide in the alveolar air. This idea was not a new one, for Traube (1871) had supposed that carbon dioxide could act by directly stimulating the pulmonary terminations of the vagi.

More recently Lumsden (1923) advanced the theory that in easy breathing the vagal endings are stimulated by the air currents passing in and out over the ciliated tracheal mucous membrane. Lumsden, however, admits that extreme distention and collapse of the lungs give rise to vagal impulses of a different kind.

It is obvious from this brief review of the literature that our knowledge of the true character of the Hering-Breuer stimulus is incomplete.

Method.

The method used is similar to the one described by Churchill and Agassiz (1926). It permits separate recording of the respiratory movements of each lung. Two brass tubes serve to divide the trachea into two separate compartments. The tubes are 14 and 22 cm. long and both have an internal diameter of 6 mm. The longer one is ligated in the bronchus on one side. The shorter extends downward to the opposite bronchus, but does not necessarily enter it. Each cannula connects with a separate respiration system including inspiratory and expiratory valves, soda lime for removing CO_2 , and a small spirometer for recording the changes in respiratory movements. Tracings of the respiratory curves were made by equipping the spirometers with writing pens. Dogs anesthetized with barbital-sodium were used, as previously described (Moore and Binger (1927)).

The operative procedure was the same in all experiments. When satisfactory anesthesia had been obtained the trachea was exposed in the neck and the right and left vagus trunks were isolated. The trachea was then opened and the metal tubes introduced and temporarily anchored in place by a ligature. After this mechanical respiration was provided separately for each lung by forcing air intermittently under slight pressure into the two tubes. The air escaped through one branch of a Y-shaped tube connected with the system. The dog was placed on the side with the forelegs extended and the thorax was opened through the 5th intercostal space, right or left as the occasion demanded. During the operative procedures within the thorax the ribs were held apart by a mechanical retractor and the lungs retracted from the field by cotton packs soaked in warm, physiological sodium chloride solution. In isolating the bronchus blunt dissection was used

as much as possible in order to lessen the danger of nerve injury. After placing a ligature beneath the bronchus the long cannula was manipulated into position, and tied firmly in place. Expansion of all lobes subsequent to this indicated that the correct position had been obtained. The further procedure depended on the nature of the experiment and will be mentioned in the descriptions of the individual experiments. Before completely closing the thorax the lungs were expanded to expel the air from the chest. Closure was accomplished by suturing the intercostal muscles. Placing of ligatures about the ribs was purposely avoided for fear of limiting the free motion of the thoracic wall. When closure was complete the opening in the trachea was made tight after the manner described by Churchill and Agassiz, and the animal connected with the two respiration systems. All animals breathed 90 to 95 per cent oxygen throughout the period of observation. During operation the animals were kept warm by an electric pad and the rectal temperature was recorded at repeated intervals. During the period of observation the animals were surrounded by warm air.

EXPERIMENTAL.

I. The Effect of Cutting One Vagus Nerve on the Respiratory Movements of Each Lung.

Schafer (1919) calls attention to the possibility of vagal section leading to a collapse of the smaller bronchial tubes from paralysis of their muscular layer. If such were the case, cutting the vagus nerve on one side might seriously interfere with the passage of air into and out of the respiratory bronchioles of the lung on the side of vagal section. Under these conditions one would expect less air to enter the lung, the vagus of which has been divided. The following experiment (No. 1) was made to test this point.

Experiment 1.—An animal, anesthetized with barbital-sodium, breathed during the control period at the rate of 28 per minute. The tidal air of the right lung was 44 cc. and the tidal air of the left lung was 56 cc. Immediately following section of the right vagus nerve the respiratory rate dropped to 14, the tidal air of the right lung rose to 55 cc., and the tidal air of the left lung to 72 cc. These figures show that unilateral vagotomy was accompanied by an increase in the tidal air of both lungs. A similar, but exaggerated response was noted after cutting the left vagus. When the left vagus was cut the right and left lungs showed an increase in tidal air to 100 and 138 cc., respectively. The experimental data are brought out in Table I and the respiratory curves are reproduced in Fig. 1.



FIG. 1. Experiment 1. Effect of cutting one vagus nerve on the respiratory movements of each lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At *A* the right vagus nerve was cut. The effect was a slowing and deepening of the respirations of both lungs. At *B* the left vagus nerve was cut. The effect was the same, but more pronounced. Time in seconds.

This experiment was repeated, and similar results were obtained.

As a corollary to these experiments, the effect of partial obstruction of the bronchus of one lung on the tidal air of each lung has been studied. The right bronchus was gradually occluded by means of a specially constructed metal tap, previously described by us (Moore and Binger, 1927) which was introduced into the system between the bronchial cannula and the respiration valves. Blalock, Harrison, and Wilson (1926) found, in their studies on morphinized dogs, that partial obstruction of the trachea in both phases of respiration causes slow, shallow breathing. In the light of these findings it was anticipated

TABLE I.

Experiment 1. The Effect of Cutting One Vagus Nerve on the Respiratory Movements of Each Lung.

Weight of dog, 17.5 kilos. Total barbital-sodium, 0.33 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	cc.
3.12	Dog breathing 90-95 per cent O ₂ throughout experiment	28	44	56
3.14		28	44	56
3.15	Cut right vagus			
3.15+		14	55	72
3.17	Cut left vagus	15	51	64
3.18+				
3.19		9	100	138

that resistance to the passage of air into and out of one lung would decrease the depth of respiration of the lung on the side of the resistance and probably increase the depth of breathing of the opposite lung. In Experiment 2, Fig. 2, during the period of resistance on the right side the tidal air of the right lung dropped from 61 to 20 cc., whereas the tidal air of the left lung increased from 79 to 116 cc. Similar results were obtained on two other occasions.

The results of the experiments in this group, then, may be summarized as follows: The effect of unilateral vagotomy on the character of

breathing is the same in both lungs, namely: There occurs an equivalent slowing and deepening of respiratory movement. This would suggest that the variation in respiratory movement that follows vagal section is not due directly to local changes in the lungs. The evidence rather supports the view that is now generally held, namely, that cutting the vagus nerves blocks peripheral impulses that reflexly modify the character of breathing. The results that we obtained are in harmony with Einthoven's (1892) experiments. Einthoven measured the changes in intratracheal pressure in anesthetized dogs when artificial respiration was provided by blowing in and sucking out a constant volume of air at each respiration. In six experiments the changes in intratracheal pressure after cutting the vagus nerves were as follows: Three animals showed a drop that was less than 1 mm. H₂O, and the other three animals showed decreases of 7, 24, and 2 mm. H₂O, respectively. Einthoven accepted these findings as evidence that section of the vagus nerves does not cause any noticeable change in the cross-section area of the bronchioles. He concluded that, when the bronchial muscles are at rest, the vagi exert little or no tonic effect upon them.

II. The Effect of Blocking One Bronchus on the Respiratory Movements Recorded by the Opposite Lung.

In four experiments, using the technique described, the bronchus to one lung was completely obstructed while obtaining graphic tracings of the respiratory movements of the opposite lung. In each instance the right bronchus was the one blocked. The result of this maneuver was a slowing and deepening of the respirations in the functioning lung. On three occasions, after a brief interval, the animals became anoxicemic and the respirations accelerated. This may be seen in Experiment 3, Fig. 3; and in Experiment 4, Fig. 4. Having established the type of response to obstruction of one bronchus we were then in a position to study the effects of this plus vagotomy on the corresponding side. This will be dealt with presently.

In the meanwhile, it should be incidentally mentioned that the response of the functioning lung was influenced by the position of the opposite lung at the time its bronchus was blocked. When the right bronchus was blocked at the end of expiration the next movement of

the left lung was a relatively deep inspiration (Fig. 4); when, on the other hand, the right bronchus was blocked at the end of inspiration, the next movement of the left lung was a relatively deep expiration (Fig. 3).

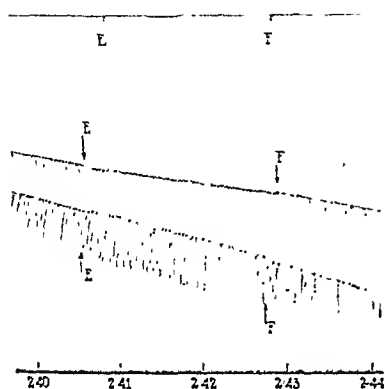


FIG. 2.

FIG. 2. Experiment 2. Effect of partial occlusion of the right bronchus on the respiratory movements of each lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At *E* the cross-section area of the top was finally reduced to 0.1 sq. cm. This caused the tidal air of the right lung to decrease from 56 to 20 cc. and the tidal air of the left lung to increase from 87 to 116 cc. At *F* the obstruction was removed and the tidal air of each lung returned immediately to the control level. Time interval 2 seconds.

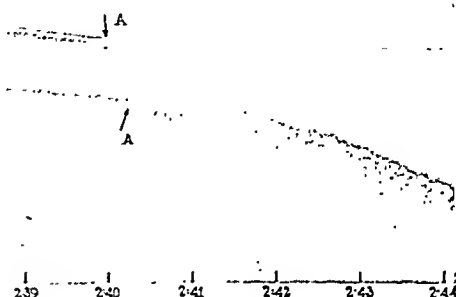


FIG. 3.

FIG. 3. Experiment 3. Effect of blocking the right bronchus on the respiratory movements recorded by the left lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At *A* the right bronchus was completely occluded at the end of inspiration. The next movement of the left lung was a deep expiration. The respiratory rate immediately slowed, but within 3 minutes accelerated to 38, 11 breaths above the control level. Time in seconds.

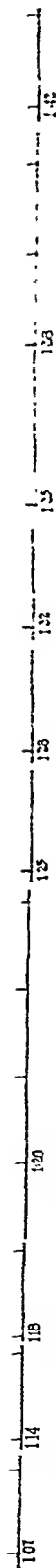


FIG. 4. Experiment 4. Effect of occlusion of the right bronchus plus right sided vagotomy on the ventilation of the left lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At .1 the right bronchus was blocked at the end of expiration. The next movement of the left lung was a deep inspiration. The respiratory rate immediately slowed, but after 5 minutes had accelerated to 34, 13 breaths above the control level. At B the right vagus nerve was cut. The respirations immediately became slower and deeper. Time in seconds.

III. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy on the Ventilation of the Left Lung.

The object of this procedure was to learn whether or not the characteristic response of unilateral vagotomy, which we have just described, is influenced by the motions of the lung, the vagus of which has been sectioned. It has previously been assumed that the Hering-Breuer reflex is set off by the alternate expansion and collapse of the lungs.

TABLE II.

Experiment 4. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy on the Ventilation of the Left Lung.

Weight of dog, 20 kilos. Total barbital-sodium, 0.32 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	cc.
1.07	Dog breathing 90-95 per cent O ₂ throughout experiment	21	86	27
1.14		22	86	30
1.18		21	86	30
1.19+				
1.20	Right bronchus blocked		0	56
1.22		26	0	159
1.25		34	0	127
1.28		35	0	114
1.32		35	0	109
1.35		32	0	109
1.38				
	Right vagus divided with scissors			
1.39		22	0	116
1.42		24	0	130

The experiments described under this heading were designed for the purpose of inquiring into this point. It is admitted that their somewhat complicated and unnatural character is objectionable, but, by them, we were able to observe the respiratory movements of one relatively normal lung while its fellow was rendered functionless by blocking its bronchus and cutting or freezing its vagus nerve. Four experiments of this type were performed, of which one is reported in

more or less detail. In two the vagus nerves were cut; in the other two frozen. Both methods gave similar results.

Experiment 4.—In this experiment, during the control period, the animal breathed at the rate of 21 per minute. The tidal air of the right lung was 86 cc. and the tidal air of the left lung was 30 cc. When the right bronchus was blocked the tidal air of the left lung increased to 56 cc. with the very next breath. 2 minutes later it had risen to 159 cc. The respiratory rate slowed immediately after the bronchus was blocked, but within 3 minutes increased to 26. 3 minutes after this the animal was cyanotic, the rate had accelerated to 34, and the tidal

TABLE III.

Experiment 5. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy on the Ventilation of the Left Lung after Division of the Right Phrenic Nerve.

Weight of dog, 11 kilos. Total barbital-sodium, 0.35 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	cc.
12.35	Dog breathing 90-95 per cent O ₂ throughout experiment	32	20	30
12.38		32	20	35
12.39	Right bronchus blocked			
12.41		38	0	41
12.44	Section of right vagus	38	0	36
12.46				
12.47		29	0	38
12.53		27	0	38
12.59		24	0	40
1 03		23	0	40

air of the left lung had decreased to 127 cc. 10 minutes later the respiratory rate was 32 and the tidal air of the left lung was 109 cc. At this point the right vagus was cut. The rate immediately dropped to 22 and the tidal air of the left lung increased to 116 cc. During the next 3 minutes the tidal air of the left lung increased to 130 cc. These changes are brought out in Table II and Fig. 4. *They show that the response to one sided vagotomy is not altered by previously preventing respiratory motions in the vagotomized lung.* This is opposed to the view which is generally held as to the nature of the Hering-Breuer reflex.

Before drawing conclusions from our own observations we must consider two possibilities which may be of importance. First, does

blocking the bronchus actually prevent lung motion? Second, could the slowing of respirations be explained on the basis of crossed innervation of the vagus fibres?

That lung motion is prevented by blocking the bronchus is probably true, since all access of air to the lung has been excluded, and since at autopsy the occluded lung presents a picture of complete collapse. It is unlikely that an atelectatic lung could follow the movements of the diaphragm and chest wall. In this connection an experiment (No. 5) was performed in which the expansion of the right lung was severely limited from the start by cutting the right phrenic nerve in the chest. Presumably the limited expansion was due to partial or complete paralysis of the right half of the diaphragm. Under these conditions the tidal air of the right lung was only 20 cc. The tidal air of the left lung was 35 cc. The respiratory rate was 32. The dog was anoxemic as indicated by the dark color of its tongue. 5 minutes after blocking the right bronchus the respiratory rate was 38 and the tidal air of the left lung was 36 cc. The right vagus nerve was cut at this point. The rate immediately slowed, but the amplitude of the respirations of the opposite lung was practically unaltered. The results of this experiment are presented in Table III. With air unable to enter the lung, and with the right half of the diaphragm paralyzed, it is difficult to conceive that there could have been any lung motion. Nevertheless, the respiratory rate slowed when the right vagus was cut. The experiment is of additional interest because it shows that slowing of respirations which follows after occlusion of the right bronchus and section of the right vagus nerve still occurs even though the phrenic nerve on the same side has been divided. This indicates that the slowing of respirations following unilateral vagotomy does not depend upon the interruption of impulses arising from the contraction and relaxation of the diaphragm.

Regarding the second possibility, the existence of crossed vagal innervation has been recognized for some time. This has recently been demonstrated for the sensory fibres by Larsell (1921).

To control the influence of crossed innervation on our results, we have devised a technique whereby the vagal fibres to the left lung were divided at the hilum. By this procedure we were able to observe the effect of right sided bronchial occlusion and vagotomy on the

TABLE IV.

Experiment 6. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy after Division of the Pulmonary Branches of the Left Vagus Nerve.

Weight of dog, 18.5 kilos. Total barbital-sodium, 0.3 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	cc.
3.13	Dog breathing 90-95 per cent O ₂ throughout experiment	22	64	64
3.17		22	66	67
3.18	Right bronchus blocked			
3.19		24	0	118
3.22		32	0	128
3.24		33	0	112
3.30	Section of right vagus			
3.33		16	0	160
3.35		19	0	155

TABLE V.

Experiment 7. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy after Division of the Pulmonary Branches of the Left Vagus Nerve.

Weight of dog, 17.5 kilos. Total barbital-sodium, 0.3 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	cc.
2.49	Dog breathing 90-95 per cent O ₂ throughout experiment	47	40	14
2.51				
2.52	Right bronchus blocked	37	0	66
2.55		43	0	66
2.58		40	0	66
3.00				
3.01	Section of right vagus	27	0	105

motions of the left lung, which had been previously denervated with respect to its vagal fibres. There could, therefore, be no question of crossed innervation. This experiment was done twice with identi-

cal results, which are presented in Tables IV and V. The upshot of these experiments was entirely analagous to that of our previous ones, showing that crossed innervation of vagal fibres was not responsible for the results obtained.

IV. *Is the Response to Unilateral Vagotomy Dependent upon an Intact Pulmonary Circulation?*

We have seen thus far that the slowing and deepening of the respiratory movements of one lung, when the vagal impulses from the other

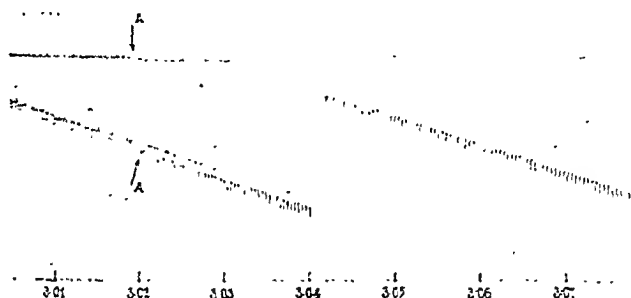


FIG. 5. Experiment 8. Effect of cutting the right vagus nerve on the respiratory movements of each lung after ligation of the right pulmonary artery. The artery was ligated within the pericardium. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. During the control period the tidal air of the right lung was 30 cc. and the tidal air of the left lung was 36 cc. At A the right vagus nerve was divided. The respirations of each lung immediately became slower and deeper. Time in seconds.

lung are intercepted, does not depend upon the motions of the vagotomized lung nor upon the existence of crossed innervation of vagal fibres. It remained to discover whether blocking the circulation of blood through the pulmonary artery of the vagotomized lung would influence the response of the other lung to vagal section. The reason for regarding this as a possibility was thought to lie in the statement encountered from time to time in the literature, and pre-

viously alluded to, that the vagal endings in the lung are locally sensitive to carbon dioxide. Indeed, a mechanism has been suggested wherein the fluctuation in tension of alveolar carbon dioxide has been conceived as the excitatory stimulus for the Hering-Breuer reflex. Adequate experimental evidence for the existence of such a mechanism is, however, not at hand. By obstructing the flow of blood through the pulmonary artery of the lung whose vagus was subsequently divided, we rendered this lung functionless in respect to its gas exchange. No normal fluctuations in the alveolar carbon dioxide tension of such a lung could occur and in time the alveolar carbon dioxide tension would be much reduced. In Experiment 8 (Fig. 5) samples of alveolar air were withdrawn from each lung a few minutes after the vagus nerve had been cut. This was accomplished by passing a small catheter deep into the bronchus and collecting the gas in sampling tubes. The air from the left lung contained 7.18 per cent of carbon dioxide, whereas that from the right, whose pulmonary artery had been ligated, contained only 1.16 per cent of carbon dioxide. It was found that vagotomy under these circumstances resulted in the same slowing and deepening of respirations recorded by the other lung, as occurred when the pulmonary circulation to the vagotomized lung was intact. This is shown in Fig. 5. It may be concluded, therefore, that the characteristic results of unilateral vagotomy do not depend upon an intact pulmonary circulation.

DISCUSSION.

In the introduction of this paper we described the experiments of several investigators to illustrate the point that a rapid respiratory rate, experimentally produced in animals, disappears immediately if the vagus nerves are divided. Our own experiences in this field had led us for some time to attach more and more importance to the local excitation of vagal nerve endings in the lungs as the most likely explanation of the rapid rates. The literature on this subject was convincing in only one respect, namely, that excitation of the pulmonary terminations of the vagus nerves is related to the alternate expansion and collapse of the lungs. The experiments that we have described are unique in having shown that the vagal influence is still present after lung motion has been prevented. In this respect they modify

our knowledge of the nervous control of breathing. The experiments are incomplete in that they do not explain the origin of the reflex that we have studied.

The inferences drawn from our experiments are sound in so far as one is willing to admit that the slowing and deepening of respirations that follow unilateral vagotomy indicate an interruption of afferent impulses that normally travel this path. One will avoid confusion in following the argument by constantly bearing in mind that the slowing and deepening of breathing after vagotomy means, we believe, that a normal reflex has been interrupted.

SUMMARY AND CONCLUSIONS.

1. Cutting one vagus nerve, while recording the pulmonary ventilation of each lung separately, has no unique effect on the ventilation of the denervated lung. Both lungs respond to unilateral vagotomy by an equivalent slowing and deepening of respiratory movement.

2. When the bronchus to one lung is blocked the first effect is a slowing and deepening of the respiratory movements recorded by the opposite lung. As oxygen want develops these movements become rapid and shallow.

3. With a combination of these two conditions, *i.e.*, when the bronchus to one lung is blocked and its vagus nerve is severed, the pulmonary ventilation recorded by the opposite lung exhibits the same changes as may result from unilateral vagotomy alone, unaccompanied by occlusion of the bronchus.

4. From these facts it may be concluded that the slowing and deepening of breathing which follows unilateral vagotomy does not depend for its occurrence upon the passage of air in and out of the bronchus of the lung whose vagus nerve has been sectioned.

5. The slowing of respirations after occlusion of the bronchus to one lung and section of the corresponding vagus nerve still occurs even though the phrenic nerve on the same side has been divided. This indicates that the slowing of respirations following unilateral vagotomy does not depend on the movements of the diaphragm on the side of vagal section.

6. When the pulmonary artery to one lung has been ligated and the vagus nerve on the same side cut, the response of the other lung is the

same as has been described, namely, its respiratory movements become slower and deeper. This is taken as evidence that the results of unilateral vagotomy are not dependent upon an intact pulmonary circulation.

7. The general conclusions from these experiments are that the slowing and deepening of respirations following unilateral vagotomy do not depend upon: (a) Passage of air in and out of the trachea. (b) Expansion and collapse of the lung. (c) Existence of a normal pulmonary circulation in the vagotomized lung. (d) Normal fluctuations in alveolar carbon dioxide tension. (e) Contraction and relaxation of the diaphragm on the side of vagotomy.

8. The slowing and deepening of respirations, alluded to, may be presumed to indicate that a normal reflex (the Hering-Breuer reflex) has been interrupted. Since this interruption occurs in spite of all the conditions enumerated under Paragraph 7, we must conclude that none of these conditions is essential to the existence of this reflex.

I take great pleasure in expressing thanks to Dr. Carl A. L. Binger for valuable suggestions, and for his helpful interest in the course of this investigation.

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EFFECT OF CERTAIN TISSUE EXTRACTS ON RED BLOOD CELL REGENERATION.*

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While most of the anemias in human beings are due to some well known cause, and are for this reason looked upon as secondary, there are instances in which the cause cannot be determined, though the blood changes indicate that even these are of the secondary type. For this reason most of the experimental work done in the past in the attempt to obtain more information as to the cause of anemia in human beings, particularly pernicious anemia, has been based on the supposition that it is due to some form of intoxication. Recently, however, evidence has been furnished indicating that the course of experimental anemias in animals, as well as of pernicious anemia in human beings, may be favorably influenced by special diets. This suggests that some of the obscure anemias may be due to the lack of some substance in the body which is essential to normal blood formation and which may be furnished in the food. The recent work showing that calcium metabolism may be affected by various means suggests that the same may be true of iron.

Experiments planned to show the effect diet may have in producing anemia in animals have not been very fruitful. Many such attempts have been made with diets poor in iron, or in some other supposed essential, but the results were usually negative. Malnutrition in animals can be easily produced, but this is never accompanied with the characteristic blood changes seen in pernicious anemia.

Usually it is thought that the oxygen requirement of the body is the most important controlling influence in determining the number of red blood cells in the circulating blood, and this belief has received

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considerable support from the experiments made on animals and human beings which had been kept at high altitudes. However, neither this work nor the work with diet proves that there may not be some other regulatory mechanism, possibly located in the liver.

In 1904 Perrin (1) reported good results in the treatment of three cases of severe anemia, which were associated with hepatic cirrhosis, by adding fresh liver to the diet. The results obtained with the feeding of liver by Whipple (2) and his associates in the treatment of experimental anemia in dogs and by Minot and Murphy (3) in the treatment of pernicious anemia, have made it evident that this organ plays an important part in red blood cell formation. Recently Whipple and Robscheit-Robbins (4) have found that water and alcoholic extracts of liver fed to dogs with long continued anemia likewise promote red cell regeneration. It will be noted that all of Whipple's experiments were made with dogs which had a long continued anemia.

The belief has been prevalent for a number of years that the spleen in the adult, through a stimulating action on the bone marrow, influences blood formation. Though this view is not accepted by all observers, it is well known that it is directly concerned in blood formation during fetal life, and particularly during adult life, in the processes of red blood cell destruction. But what is the evidence in support of the belief that the spleen produces an internal secretion which regulates the number of red blood cells in the circulation? For a detailed discussion of the functions of the spleen the reader is referred to the recent papers by Eddy (5) and Krumbhaar (6).

Brinckmann (7) found that feeding normal rabbits with fresh ox spleen caused a reduction in the red and white blood cells. Zalinski (8) and Danilewski (9) reported a marked increase in the number of red cells by a single intraperitoneal injection of splenic extract, but Patou, Gulland and Fowler (10) were unable to confirm these observations. Krumbhaar and Musser (11) also report an increase in the red count following the injection of a fresh splenic extract. This increase lasted 1 or 2 days. Eddy (5) in his experiment used saline extracts of powdered dried spleen and protein-free extracts of fresh and dried spleen. The red cell counts were made at half hour intervals and continued 4 to 5 hours after the injection. Each of his preparations caused a decrease in the number of red cells in the circulation. On the other hand, Leake and Bacon (12) report that the feeding of fresh spleen, or the intravenous injection into rabbits of saline solution extracts of fresh spleen, caused a fall in the number of circulating erythrocytes within 24 hours. It went to 7 per cent above normal after the 3rd day, but soon fell to normal when administration was discontinued.

Methods.

Extracts of liver, spleen, gastric mucosæ and hemoglobin solution were used in the experiments. The gastric mucosæ were obtained from pig stomachs, and the

other organs from rabbits or calves. For preparing the protein-free extracts the tissues were ground to a pulp by passing repeatedly through a meat grinder. They were then extracted with an equal volume of 95 per cent alcohol to which had been added 0.5 per cent acetic acid. After standing overnight in the ice box the mixture was passed first through gauze and then through filter paper until the filtrates became clear. The tissue remaining was extracted twice in the same manner with 75 per cent alcohol. The filtrates from the three extractions were combined and evaporated to dryness at a temperature not exceeding 45°C., under negative pressure. The partially dried mass was then extracted with 75 per cent alcohol, filtered and the filtrate again evaporated to dryness. For one experiment, Dr. Zucker, of this department, kindly prepared for us a fat-soluble non-saponifiable extract of the liver.

In addition to the above, simple saline extracts of the tissues were used. These extracts were prepared by grinding up the fresh organ with saline solution in a mortar. Ten parts of saline solution were used to 1 of the tissue. The mixture was kept in the ice box overnight and then cleared either by filtration through sterile filter paper or by centrifugalization. The hemoglobin solution was made by laking 15 parts of washed rabbit red cells with 25 parts distilled water and cleared by centrifugalization.

Rabbits were used in all experiments. They were made anemic by bleeding from the heart; ether anesthesia was used. If necessary to save the life of the animal, the blood lost was replaced with saline solution. The blood removed constituted about 3 per cent of the body weight, or about 60 per cent of the total volume. Several blood counts, hemoglobin determinations and reticulated cell counts, were made before the bleeding and at frequent intervals afterwards. At the end of each experiment autopsies were done, particular attention being paid to the organs concerned in blood destruction and regeneration. The details of the autopsy findings will be reported subsequently.

The normal red count in our rabbits was found to be between 5 millions and 6 millions, which the bleeding usually lowered to about 2.5 to 3 millions. Only those animals with low red cell counts were used in the experiments. The animals were all kept under the same conditions as to light, food and temperature, and were weighed regularly. Our interpretation of the results of the injection of the different substances was based upon the rapidity with which the normal count was restored, as compared to a series of similar untreated animals. Each of the experiments has been repeated several times during the last few years.

In our preliminary experiments we found that the red count in the control animals losing about 60 per cent of their total blood was usually

not restored to normal until about 3 to 4 weeks subsequent to the operation, though evidence of regeneration became quite evident at the end of the 2nd week. There was also a marked loss in weight during the first 2 weeks. With the increasing red count there was an increase in reticulocytes.

The first experiment was planned to show the effect of protein-free extracts of the liver, spleen and stomach on normal animals. Two rabbits were used for each extract. The freshly prepared extract in doses of 5 mg. per kilo of body weight was given subcutaneously on 6 successive days in each week. As no effect was apparent after the 1st week, the dose was then increased to 10 mg. and this continued for a period of 25 days.

The animals after receiving the liver extract showed some increase in red cells after 30 days, but the increase was so slight that it was within the limits of normal variations in the red cell count. There was an increase in the neutrophilic leucocytes. Those receiving the stomach extract showed a very slight decrease. On the other hand, the rabbits receiving the spleen extracts showed a decrease of 1.8 and 3 millions, respectively. The number of reticulocytes was increased in the animals receiving the liver extracts, but was not affected by extracts of spleen and stomach. There was some increase in the lymphocytes.

This experiment was repeated several times with the same results as regards the liver and stomach extracts. In subsequent experiments, however, the spleen extract, whether protein-free or from freshly prepared cell emulsions in salt solution, was more active in causing a decrease in the red cell count. In the animals receiving the spleen extracts the per cent of reticulocytes, regardless of the degree of anemia, was always low, sometimes considerably below normal. There were no changes in the white blood cells which could be ascribed to the extracts.

The preceding experiment was repeated with animals which had been made anemic by one large bleeding. 2 days subsequent to the bleedings the red cell counts in this series of animals were between 2.5 and 3.2 millions. Three were left as controls, while the others received 10 mg. doses of the extracts of liver, spleen and stomach. All injections were made into the subcutaneous tissues.

Blood regeneration in the animals receiving the liver extract appeared to be a little more active than in the controls, and the reticulocytes increased earlier and were somewhat more numerous. The neutrophil leucocytes also were somewhat increased. The extracts of the gastric mucosæ appeared to exert no influence on regeneration. In the animals receiving the spleen extract there was very little red cell formation. When small doses were given there was a prolonged period during which there was little evidence of regeneration; but with larger doses the red count was still further depressed. The number of reticulocytes in these animals was very low.

A new series of rabbits was made anemic by bleeding from the heart and then injected with extracts, both saline and protein-free, prepared from the liver and spleen of rabbits which had been made anemic by repeated bleedings. The animals from the organs of which the extracts were prepared were bled every other day for 18 days. During this period a little more than 200 cc. of blood was taken from each animal, so that the final red cell count in each was a little less than 2 millions, and the hemoglobin approximately 20 per cent. The average number of reticulocytes for the four animals in the beginning was .5 per cent; after 13 days 7 per cent, and after the final bleeding, approximately 21 per cent. The animals were bled to death under ether anesthesia and the organs used for the preparation of extracts. Protein-free extracts were prepared from the livers of these animals. Because of their small size we were compelled to rely on saline extracts of the spleens. From the blood obtained at the last bleedings, hemoglobin extracts were prepared. The methods used in preparing these extracts have been described.

The hemoglobin solution was injected every other day into the peritoneal cavity of the rabbits in doses of 10 cc. The spleen extract was given in doses of 2 cc. and the liver extract in doses of 10 mg. per kilo, both subcutaneously.

Again, in this experiment, the spleen extract rabbits showed practically no evidences of any ability to generate red cells. The reticulocytes were not increased above the normal, which suggests that the effect noted above may be due to an inhibiting action which the spleen extracts have on blood regeneration, rather than a destructive action, which we at first thought probable.

On the other hand, the rabbits receiving the hemoglobin and liver extracts did show active red cell regeneration by the end of the 2nd week, and the per cent of reticulocytes was somewhat higher than that seen in the controls for the corresponding period.

In the next experiment we tested the action of a fat-soluble non-

saponifiable liver extract kindly prepared for us by Dr. Zucker of this department. In addition, we again studied the action of the protein-free extracts of the liver and spleen. This spleen extract had been kept in the ice box for more than 5 months.

The results of this experiment indicated that the fat-soluble non-saponifiable liver fraction was inactive, while the spleen extract, in spite of its age, showed the usual inhibiting effect. The blood regeneration in the rabbits receiving the non-protein liver extract was quite active.

Though none of the extracts used appeared to be very active in stimulating blood regeneration, yet there was some indication that the normal red cell count was restored a little more quickly in the animals which had received the liver extract and hemoglobin solution than in the untreated anemic controls. As extracts prepared from the organs of animals made anemic by bleeding were inactive, our next experiment was made with extracts prepared from the organs of animals made anemic by destroying the red cells within the body. This was accomplished by giving intravenous injections of distilled water. The injections were given five times in 2 weeks. One animal received 140 the other 205 cc. of water. The purpose of the experiment was to see if the organs of animals made anemic in this manner would contain the supposedly stimulating end-products of the red cells. Briefly, the results of the injection of these extracts showed no difference from the preceding.

While preparing these animals we were surprised to note the amount of distilled water which could be given in this manner without causing death. In subsequent experiments it was found that rabbits would frequently survive doses of 80 cc. and 120 cc.

DISCUSSION.

In part of our experiments with liver extracts we obtained results which indicated that they contained active substances, but while red cell regeneration began early in all the liver extract animals, a few of the controls showed the same early regeneration. In addition, the differences were not striking in any instance and were within the limits of experimental error. For this reason they were repeated several times over a period of more than 3 years, with the results mentioned.

Of the different extracts used, those prepared from the livers of anemic animals acted best, as the red count in the rabbits receiving these preparations apparently was restored to normal more rapidly than in the controls, and the reticulocyte count was definitely higher.

On the other hand, the effect of the various spleen extracts was quite striking. Where the amounts given were small, the regeneration of blood was much slower than in the control animals; while with large doses there was a further decline in number of red cells. The reticulocytes were always much fewer in number. It is possible that we

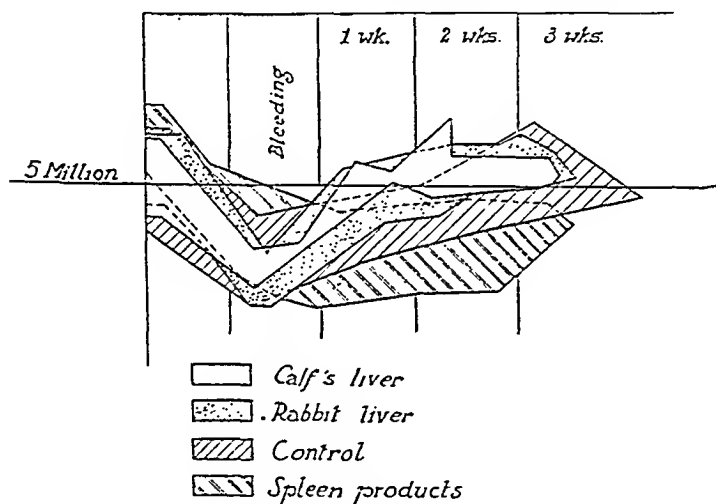


CHART 1.

were dealing with a poison, but the low reticulocyte count suggests inhibition of red cell formation rather than cell destruction.

In the body of the paper we have given no tables showing red cell counts, hemoglobin, etc. Had the results been more positive in character, they would have been included, but under the circumstances it seemed unnecessary to do so. The chart shows more graphically the results we obtained in our experiments with liver and spleen extracts, and represents the averages obtained in numerous experiments. It suggests that blood regeneration in the animals which received both rabbit and calf liver extracts is more active than in the controls, but

the difference is not marked. On the other hand, the spleen obviously has an inhibiting effect.

Our results are particularly interesting in view of those obtained with the feeding of liver by Whipple in the experimental anemias of dogs, and by Minot and Murphy in pernicious anemia. Several explanations of the discrepancies are possible. First, rabbits may not respond to the liver treatment; second, the active substance may have been destroyed by the heat used in drying our alcoholic extracts, though it did not exceed 45°C.; third, our preparations were given by subcutaneous injection, their's by mouth; fourth, our animals were made anemic by a single large bleeding, while Whipple's experiments were conducted with animals having a chronic anemia produced by repeated bleedings.

It seems not improbable that the marrow of animals with a long continued anemia will be hyperplastic and react more readily to the presence of a stimulating substance than those with an acute anemia produced by a single large bleeding. In the former case the marrow is prepared to act, in the latter case it is not.

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STUDIES ON THE MODE OF SPREAD OF *B. ENTERITIDIS* MOUSE TYPHOID INFECTION.

I. NATIVE EPIDEMICITY.

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GENERAL INTRODUCTION.

Our experimental studies of epidemics have been confined thus far to two native animal diseases: mouse typhoid, caused by an organism of the animal paratyphoid B group, *B. pestis caviæ*,¹ and a respiratory infection of rabbits, *Bact. lepi-septicum* snuffles and pneumonia. Mouse typhoid, being readily adapted to laboratory manipulations, has yielded data bearing upon its prevalence and mode of spread; rabbit snuffles, notwithstanding the technical difficulties encountered, has been of use in confirming the data and extending their application (1, a).

The scope of experimental epidemiology may be enlarged by extending the investigations to still other animal diseases. With this end in view, we have turned our attention to a third native infection, a variety of mouse typhoid induced by a bacillus of the enteritidis group. This infection is wide-spread among rodents (2), and occurs also in man as a form of food poisoning (3). Mice affected show gross and microscopic lesions resembling those occurring in the paratyphoid B infection (4). Indeed, the major features of these two diseases in mice are similar.

However, two rather special characteristics of the enteritidis infection in mice make a study of the disease of special interest from the point of view of experimental epidemiology. The first of these is the fact that a high titre bacteriophage may be obtained readily from a large per cent of infected animals; the second is that under relatively

¹ This organism is also designated *B. aertrycke* and *B. paratyphi*.

simple conditions, the typical smooth colony forms of *B. enteritidis* change to mucoid and rough colony variants. Similar bacteriophage and transformation processes have been considered, of late, to play such an important part in determining the prevalence of other diseases, that it seemed desirable to study the phenomena as they occur in *B. enteritidis* mouse typhoid infection and determine, if possible, the extent to which they influence its spread.

The studies to be described are divided as follows: (1) Bacteriological tests on mice from various scattered populations to determine the prevailing colony types of paratyphoid-enteritidis bacilli. The results of these tests are summarized in this paper. (2) Attempts were made to determine the conditions under which "spontaneous" transformations of colony types occurred. These experiments are described in the next paper. (3) In Papers III and IV of the series, comparative studies of bacilli from the various colony types were carried out with special attention to differences in virulence. From these results, a working hypothesis was formulated to explain the mechanism of colony transformation. (4) Finally, an elaborate study of several populations involving several thousand mice spontaneously infected with *B. enteritidis* and a Friedländer-like organism has been carried on over a period of 2 years, to determine at various interepidemic, preepidemic, and epidemic periods, the colony types and relative virulence of the specific bacteria, and the effect of changes in carrier rate, season, and experimental alteration in population resistance on the spread of disease. These results are to be published later.

Types of B. enteritidis Recovered from "Spontaneously" Infected Mice.

Apparently there is general agreement among recent investigators that in native paratyphoid-enteritidis infection, the smooth colony type of organism predominates at all times.

Thus Topley encountered only smooth types of *B. gaertner* and *B. aertrycke* during his observations of experimental mouse typhoid epidemics. Thomas describes paratyphoid-enteritidis bacilli isolated from guinea pigs as forming, in general, the smooth type of colony, although one strain of *B. enteritidis* appeared rough (2, c). Savage and White, in an extensive investigation of the *Salmonella* group, state that "in direct platings from pathological material, colonies of

Salmonella bacilli are, as a rule, wholly smooth. On occasion, however, roughness may be detected in these first cultures" (3, d). Nelson, describing a typhoid epidemic among guinea pigs, states that smooth colony types predominated; occasionally, a few mucoid variants were encountered.

During the past 5 years, one of us (Pritchett) has tested a number of batches of mice obtained from various sources to detect the presence of mouse typhoid bacilli. The results of these tests, which include the species and colony type of bacillus obtained, are summarized briefly in Table I and may be stated as follows:

Group 1. Hagedoorn Albinos.—10 mice received from Professor Hagedoorn of California in June, 1922. 1 was dead on arrival and at autopsy *B. enteritidis* was recovered from the blood and feces. A year later a mouse of this strain, a descendant of the original 10, died and was found at autopsy to harbor the smooth colony type of *B. enteritidis*.

Group 2. Baltimore Albinos.—100 mice received from a dealer in Baltimore. These mice had been strain inbred for a year and a half. 1 mouse was dead on arrival and other mice died at intervals of a few days. The smooth type *B. enteritidis* was cultured from the blood and feces of the 10 or 12 autopsied.

Group 3. Annandale Hybrids.—Composed of black, brown, yellow, and white mice. 51 of these mice were received from Annandale, New York, in November, 1922. 3 were dead on arrival, and 1 other died the next day; all were found to harbor *B. pestis caviæ* in the blood and feces. Of the 47 which lived, 12 were found to be fecal carriers of *B. pestis caviæ* so that of the entire group about 23 per cent were infected with this organism; smooth colony types alone were recovered.

Group 4. Bagg Albinos.—Of 45 mice sent from Cold Spring Harbor, New York, in December, 1922, 1 was dead on arrival and was found to harbor *B. pestis caviæ* in the blood, feces, and internal organs. Of the 44 which lived, 36 were fecal carriers of *B. pestis caviæ*, so that 82 per cent of the original lot of 45 were shown to be infected with this organism; only smooth types were obtained.

Group 5. Lathrop Blacks.—45 mice sent from Cold Spring Harbor, New York, in December, 1922. Two separate fecal cultures of all the mice failed to show any mouse typhoid bacilli.

Group 6. Lathrop Browns.—100 mice sent from Cold Spring Harbor, New York, in the early spring of 1923. 1 mouse was dead on arrival and 10 or 12 succumbed during the 1st week; all showed the presence of smooth colonied *B. pestis caviæ* in the blood and feces.

Group 7. Louvain Albinos.—7 mice were received from the University of Louvain, Belgium, in May, 1924, of a stock bred in that laboratory for 3 or 4 years. By the end of May, all but 1 had died, and the single survivor died later. From three autopsies heavy growths of smooth type *B. enteritidis* were obtained from the spleen and feces.

TABLE I.
Prevalence of Mouse Typhoid Bacilli in Different Mouse Populations.

Group	Description of mice examined	Source of mice and date of examination	No. in lot	Percent negative for mouse typhoid	Mouse Typhoid I (<i>B. enteritidis</i>)			Mouse Typhoid II (<i>B. pestis carae</i>)		
					No. of positive	No. of smooth colonies	No. of mucoid colonies	No. of positive	No. of smooth colonies	No. of mucoid colonies
1	Hagedoorn albinos	California, 1922	10	?	1 mouse	1 mouse	0	0	0	0
2	Albinos	Baltimore, 1922	100	?	10 mice	10 mice	0	0	0	0
3	Hybrids, black, brown, yellow, and white	Annandale, N. Y., 1922	51	77	0	0	0	23 per cent	All	0
4	Bagg strain albinos	Cold Spring Harbor, N. Y., 1922	45	18	0	0	0	82 per cent	All	0
5	Lathrop strain black	Cold Spring Harbor, N. Y., 1922	55	100	0	0	0	0	0	0
6	Lathrop strain brown	Cold Spring Harbor, N. Y., 1923	100	?	0	0	0	10 mice	10 mice	0
7	"Louvain" albinos	Louvain, Belgium, 1924	7	?	3 mice	3 mice	0	0	0	0
8	Rockefeller Institute farm albinos	Princeton, N. J., 1923	100	?	10 mice	10 mice	0	0	0	0
9	Rockefeller Institute "Lynch" black agoutis	New York, N. Y., 1922	50	76	22 per cent	All	0	2 per cent	0	0
10	Albinos	Pennsylvania, 1925	64	88	22 per cent	100 per cent	0	0	0	0
11	Rockefeller Institute breeding room albinos	1922-1926	Many thousands	?	Less than 0.01 per cent	All	0	0	0	0

12	Pritchett experimental epi- demic	1925	80	60	Spontaneous 37.5 per cent in- fection	96.4 per cent	14.2 per cent	2.5 per cent experi- mental infection	0
13	Webster experimental epi- demics	1925-1927	4000+		Spontaneous M. T. I infection	99+	1 per cent or less	0	0

Group 8. Princeton Albinos.—100 mice received from the Department of Animal Pathology in October, 1923. 1 mouse was dead on arrival, but autopsy cultures showed no mouse typhoid bacilli. Within 2 or 3 days others died and showed the presence of smooth *B. enteritidis* in the blood and feces.

Group 9. Lynch Black Agoutis.—50 mice obtained from The Rockefeller Institute cancer stock in November, 1922. Three fecal cultures were made on the 50 mice between November 8th and December 4th. 11 (22 per cent) were found to be fecal carriers of smooth *B. enteritidis*, while 1 mouse carried smooth type *B. pestis caviæ*.²

Group 10. Pennsylvania Albinos.—64 mice obtained from a Pennsylvania breeder in October, 1925. The mice began to die immediately, about 50 per cent succumbing in the first 10 days after arrival. 22 per cent of these mice showed the presence of smooth *B. enteritidis*, either in the feces during life or in the blood and organs at autopsy.

Group 11.—The mouse breeding room at The Rockefeller Institute, New York, supplies 500 to 1000 mice a month. The population of this room is entirely free from *B. pestis caviæ* infection, so called Mouse Typhoid II, but does yield an occasional carrier of the smooth type of *B. enteritidis*, Mouse Typhoid I. Estimates of one 6 month period showed the percentage incidence of carriers to be less than 0.01 (1, b).

Group 12.—A group of 80 mice which had survived a series of experimentally induced epidemics of *B. pestis caviæ* was found at autopsy to be infected with both the paratyphoid ($2\frac{1}{2}$ per cent) and the enteritidis organisms ($37\frac{1}{2}$ per cent). All the *B. pestis caviæ* colonies were smooth; while of the *B. enteritidis* infected animals, 14.2 per cent showed an occasional mucoid colony on the culture plates (1, b).

Group 13.—Finally, from groups of mice totalling over 4000 in number, among which *B. enteritidis* typhoid epidemics have been occurring, there have been recovered from autopsy cultures, with few exceptions, only smooth colony types (see introductory Paragraph 4, Division 4).

SUMMARY.

Thirteen batches of mice from nine different sources were tested for the presence of mouse typhoid bacilli. Individuals from nine of the groups were found to be infected with the *B. enteritidis* type, four with the paratyphoid B, one with both, and one with neither type. With two exceptions, smooth type colonies alone were found. These results are in conformity with similar observations

² The population from which these mice were drawn is entirely separate from the regular Institute breeding room. Lynch has already reported the occurrence of two mouse typhoid epidemics among the cancer stock, that of 1918-19 being due to *B. enteritidis*, and that of 1920-21 to *B. pestis caviæ* (2, c).

as reported by others and confirm the general belief that smooth colony types of paratyphoid-enteritidis bacilli prevail throughout the various stages of rodent typhoid infection.

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STUDIES ON THE MODE OF SPREAD OF *B. ENTERITIDIS* MOUSE TYPHOID INFECTION.

II. EFFECTS OF EXTERNAL CONDITIONS ON THE OCCURRENCE OF SMOOTH, MUCOID, AND ROUGH COLONY TYPES.

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PLATE 33.

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The observations on the prevalence of various bacterial types in spontaneous mouse typhoid infection (1, *a*) indicate that the smooth colony forms of paratyphoid-enteritidis bacilli are most common during all the various stages of the disease and the presence of a mucoid or rough colony variant is an exceptional occurrence. Hence it is questionable whether such bacterial transformations, or indeed any bacteriophagic phenomena, influence the extent and severity of the disease. However, this general subject has received so much attention of late in the studies of d'Hérelle (2), Arkwright (3), Hadley (4), and others, that it seemed necessary to study further the problems involved. It is intended in this paper to describe certain conditions under which the transformations of *B. enteritidis* occur from smooth to variant colony forms, and from variant back to smooth type colonies; and later, to offer a partial explanation of the nature of the process.

Cultures were obtained from spontaneously infected mice from various sources (1, *a*). One of these strains (No. 3) has been studied by Amoss and Haselbauer (5), Thomas (6), and Sakai (7), and placed in the enteritidis group.¹ But, chief attention has been given to

¹ This same culture has been used by Theobald Smith and Nelson (*J. Exp. Med.*, 1927, xlv and xlv) in identifying organisms from an epidemic of guinea pig typhoid. The mouse typhoid (M. T. I) strain used by them for purposes of identification is our Strain 3. They have designated it *B. paratyphi*, whereas we consider it to belong in the *B. enteritidis* group.

strains derived from certain populations of mice at The Rockefeller Institute, among which mouse typhoid has prevailed during the past 2 years in endemic or epidemic form (1, *a*, Group 13). The cultures were quite similar to one another and to those from outside sources (1, *a*, Table I). In brief, they consisted of Gram-negative, motile rods, growing diffusely in broth, fermenting dextrose, maltose, xylose, rhamnose, and mannite, but not lactose, saccharose, salicin, and inosite. No indole was produced; lead acetate was blackened. Stock *B. enteritidis* sera agglutinated the strains to titre and sera prepared from several of our own strains brought about maximum agglutination in all.

Colony Formation.

Direct Plating from Autopsy Material or from Fecal Carriers.—As stated previously (1, *a*, Group 13, Table I), several thousand mice have been examined bacteriologically during the course of our observations on special mouse populations, and found to harbor *B. enteritidis*. The cultures, when taken directly from the animal, streaked over plain agar plates, and incubated at 37°, formed typical smooth colonies. Occasionally, however, if the plates were left at room temperature—23°C.—for 12 hours or more at any time during the period of observation, mucoid colonies developed, or the smooth forms became surrounded by a ring of mucoid growth. Still more rarely and under different circumstances, an occasional rough colony was encountered.

Serial Passage of Smooth Colony Types on Agar and in Broth.—The natural tendency for *B. enteritidis* to form smooth type colonies can be interfered with in many ways.² But in order to analyze the transformation process as it occurs “spontaneously,” we decided to observe the relatively simple conditions which might be encountered normally in nature and in the laboratory.

Experiment 1 shows the effect of direct agar passage at 37° on the type of colony formation.

Experiment 1.—Four mouse strains of *B. enteritidis* were taken directly from the stock meat infusion agar slant and passed serially on meat infusion agar

² In Hadley's monograph, the literature on this subject is reviewed at length (4).

plates, pH 7.4. Strain 1 is a single cell culture³ obtained on December 2, 1925, from a mouse from one of the special populations mentioned above (1, *a*, Group 13). Strain 2 is a similar culture from another mouse dying on the same date. Strain 3 is a single cell culture obtained from a wild mouse found dead in a Rockefeller Institute laboratory in 1921. This strain has been studied by other investigators (5-7). Strain 4 is a single cell culture obtained in August, 1925, from a mouse from one of the special populations (1, *a*, Group 13).

A small amount of the stock culture was taken from each slant and spread over plates containing plain beef infusion agar. After 24 to 48 hours incubation at 37°, the resulting colonies were examined, and one of them picked and spread over another agar plate. This method of passage was carried on eight times for each strain.

TABLE I.

Passage of Smooth Type Colonies of B. enteritidis on Plain Agar Plates at 37° and 23°C.

Date	Passage	Temperature °C.	Strain 1		Strain 2		Strain 3		Strain 4	
			Colony type seeded	Colony type resulting	Colony type seeded	Colony type resulting	Colony type seeded	Colony type resulting	Colony type seeded	Colony type resulting
1926										
11/15	1	37	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
		24	"	"	"	Mucoid	"	"	"	"
11/17	2	37	"	"	"	Smooth	"	"	"	"
		24	"	"	"	Mucoid	"	"	"	"
11/19	3	37	"	"	"	Smooth	"	"	"	"
		24	"	"	"	Mucoid	"	"	"	"
11/21	4	37	"	"	"	Smooth	"	"	"	"
		24	"	"	"	Mucoid	"	"	"	"

Eight agar passages of these four single cell, smooth colony strains had no modifying effect on colony type. Plates from each passage showed 100 per cent typical smooth colony forms. Other mouse strains of *B. enteritidis* behaved in a similar manner for as many as twenty passages.

The effect of temperature on colony type formation was then tested (Experiment 2).

³ Single cell strains were obtained by the use of a technique described by Reimann (8).

Experiment 2.—The cultures used in Experiment 1 were passed in the same manner as before, except that each transfer was made in duplicate. One set of plates was incubated at 37°C.; the other left at room temperature, 23°. A single, well isolated colony from the 37° plate was used for each transfer. The results are shown in Table I.

Strains 1, 3, and 4 remained 100 per cent smooth at both 37° and 23° for four passages. "Smooth" colonies of Strain 2, however, when incubated at 37°, grew out 100 per cent smooth, but when allowed to grow at room temperature at 23°, appeared entirely mucoid. These colonies remained mucoid when transferred at 23°, but invariably reverted to smooth forms when grown at 37°. In all, twenty-one cultures of *B. enteritidis* were tested in this way. Fourteen came from the populations described above (1, *a*, Group 13), four from outside mice, and two from spontaneous guinea pig infections. Three of these twenty-one strains showed this characteristic of smooth growth at 37° and mucoid growth at 23°.

D'Hérelle (2) and Hadley (4) mention a number of investigators who have studied the effect of temperature on bacterial transformation, and more recently, Knorr (9) states that certain paratyphoid strains grown in broth at 22° and transferred to enriched solid media of a certain thickness and pH of 8.0 result in the appearance of mucoid colonies after 24 to 48 hours. However, we have seen no definite experimental evidence relating mucoid colony formation directly to temperature conditions.

From these experiments it is concluded that mouse strains of smooth type *B. enteritidis* tend to remain type-pure if passed from agar to agar at 37°, but that an occasional smooth strain becomes mucoid if grown on agar at 23°. The resulting mucoid colonies will remain so indefinitely if transferred and grown at 23°, but when the temperature is raised to 37°, revert immediately to the smooth form.

True rough colonies were not found on any plates during the serial agar transfer of twenty-one smooth colony strains. Fresh stock cultures were passed, together with others, 2, 4, 8, and 12 weeks, and 2, 4, and 6 months old. Colonies 72 hours and 3 weeks old were also used. Dry plates and plates with considerable surface moisture were tested. Frequently, the dry plates produced atypical rough

colonies after 24 hours incubation, but these became relatively smooth at 48 hours, and when transferred, grew diffusely in broth and appeared entirely smooth on fresh agar plates.

From old broth cultures, however, true rough colony types were obtained.

Experiment 3.—Twenty smooth type mouse typhoid strains of *B. enteritidis* were studied. Strains 1, 2, 3, and 4 have been described in the first experiment. Nos. 5, 6, 7, and 13 to 20 are single cell cultures, obtained from the special populations from July, 1925, to April, 1926. Nos. 8, 9, 10, and 11 came from outside mice dying from "spontaneous" mouse typhoid (1, a). These are single colony cultures obtained after repeated isolation. Strains 20 and 21 came from cases of spontaneous guinea pig typhoid.

TABLE II.
Transfer of Smooth Colony Strains from Broth to Agar.

Date	Age	Strain No.																				
		1	2	3	4	5	6	7	8	9	10	11	13	14	15	16	17	18	19	20	21	
	days																					
3/22	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
3/26	12	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
3/28	14	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
4/11	28	S	S	S	R	R	S	R	S	S	S	S	R	R	R	S	S	R	R	S	S	
4/16	33	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	R	R	R	R	R	

S = smooth colony. R = rough colony.

Each culture was transferred from the stock agar slant to 5 cc. centrifuge tubes of beef infusion broth, pH 7.4. After 17 hours incubation at 37°, they were placed at room temperature. 8 days later a drop of sediment from each tube was streaked over the surface of 15 cm. Petri dishes containing plain meat infusion agar. The plates were incubated for 48 hours, and then left at room temperature for 1 week. Similar platings were made on the 12th, 14th, 28th, and 33rd days. All suspected rough colonies were grown in broth to detect the characteristic granular type of growth, transferred to plain agar, grown in sugars, and identified by means of specific sera of a titre of 1:10,000. The results are shown in Table II.

No rough colonies were seen on the 8th, 12th, or 14th day. At 28 days, however, and again at 33, several strains, when transferred to agar, showed numerous typical rough colonies agglutinating in

B. enteritidis serum. Transfers from a single colony to broth grew granularly and three serial passages of single colonies from agar to agar resulted in pure rough type cultures.

When single colonies of the rough variant strains are passed from agar to agar, they remain pure for several transfers, but eventually revert to smooth. When passed from broth to broth, reversion to the smooth type occurs after the first or second passage.

Mucoid and rough colony variants also occur when broth cultures of the smooth colony forms are transferred daily.

TABLE III.

Passage of Smooth Type Colonies of B. enteritidis in Plain Broth at 37°C.

Strains	Colony type seeded	Colony types resulting	Passage								
			1 12/21 1925	2 12/22	3 12/23	4 12/24	5 12/26	6 12/27	7 12/29	8 12/30	9 1/7 1926
No. 1	Smooth	Smooth	++++	++++	+++	+++	+++	+++	++	++	+++
		Mucoid	0	0	+	+	+	+	++	++	+
		Rough	0	0	0	0	0	0	0	0	0
No. 3	"	Smooth	++++	++++	++++	++++	+++	+++	+++	+++	+++
		Mucoid	0	0	0	0	+	+	+	1	1
		Rough	0	0	0	0	0	2 colonies	0	1	1

++++ = 100 per cent; +++ = 75 per cent; ++ = 50 per cent; + = 25 per cent; 1 = 10 per cent.

Experiment 4.—Single cell Cultures 1 and 3 were employed. Both were seeded from the stock strain to beef infusion broth at pH 7.4, and incubated at 37°. After 24 hours, one loop of culture was streaked over the surface of an agar plate and a second loop transferred to a fresh broth tube. 24 hours later the culture in the latter tube was plated and transferred again. In all, nine broth passages and nine sets of agar plate transfers were made. Table III shows the results.

Strain 1 showed only smooth type colonies on agar transfer plates until the third broth passage. Mucoid colonies then appeared in gradually increasing numbers, due probably to the fact that after 24 hours incubation, the plates were left at room temperature. After four passages of Strain 3, mucoid forms appeared, later accompanied by occasional rough colonies.

To summarize briefly it may be stated that (1) most smooth type cultures of mouse typhoid *B. enteritidis* remain smooth when passed on agar; (2) that an occasional smooth strain becomes entirely mucoid when grown on agar at 23° and changes again to smooth when the temperature is raised to 37°, and finally (3) that when smooth types are grown in broth for 3 weeks or more, or transferred daily from broth to broth and plated on agar, rough colonies appear which remain rough for several agar passages, but revert to smooth forms when grown in fresh broth. Apparently, therefore, *temperature* is one factor involved in this kind of reversible transformation from smooth to mucoid colony, and a *fluid state of the media* a factor influencing the reversible transformation from smooth to rough colony form.

Effect of Bacteriophage on Colony Formation.—Many investigators have noted that bacteria grown in the presence of an active lytic agent give rise to secondary cultures which form mucoid and rough colony variants. Indeed, there seems to be general agreement with Arkwright's statement that bacteriophage stimulates normal microbic mutation (3). The results of our own observations, however, while agreeing with the general findings of previous investigators, suggest a different explanation of the process.

Active bacteriophage was obtained by various procedures,—namely, broth passage and filtration of a smooth, "eaten" colony of *B. enteritidis*, taken directly from an autopsy plate, and passage and filtration of smooth type cultures plus mouse intestinal material. The titre of this bacteriophage was found to be 10^{-9} for all of our single cell, smooth colony types of *B. enteritidis*.

Experiment 5 was planned to show the effect of bacteriophage-containing agar on the colony formation of smooth type strains of *B. enteritidis*.

Experiment 5.—300 cc. of agar in flasks were melted, cooled to 48°, and poured into 10 cm. Petri dishes. The agar used for the control plates received 30 cc. of plain broth, pH 7.4, just before pouring; a second flask, 30 cc. of 10 per cent bacteriophage in broth, and a third, 30 cc. of undiluted bacteriophage in broth (titre 10^{-9}).

A small portion of each bacterial culture was taken directly from the stock slant and spread over the surface of two plates each, of plain agar, 1 per cent and

TABLE IV.
Passage of Smooth Type Colonies of *B. enteritidis* to Plain and Phage Agar at 37° and 23°C.

Date	Temperature	Culture used for inoculation			Colony types appearing on plates		
		Strain	Colony type	Source	Plain agar	Agar + phage 1 per cent	Agar + phage 10 per cent
1926 11/23	37	1	Smooth	Stock slant	SS + + + + +	SR + + E + +	SR + + E +
		2	"	"	SS + + + + +	SR + + + + +	SR + + +
		3	"	"	SS + + + + +	SR +	SR + Ring +
		4	"	"	SS + + + + +	E Ring +	Ring 2 colonies 0
23		1	"	"	SS + + + + +	0	0
		2	"	"	MS + + + + +	MR + +	M +
		3	"	"	SS + + + + +	E 1 colony Ring +	SR + M +
		4	"	"	SS + + + + +	MR 4 colonies	Ring + M +
12/9	37	5	"	"	SS + + + + +	SR + E +	SR + E + M +
		6	"	"	SS + + + + +	SR + E +	SR + E + M 1 colony
		7	"	"	SS + + + + +	SR +	SR +
		8	"	"	SS + + + + +	SR +	SR + E + M +
23		5	"	"	SS	SR +	SR +
		6	"	"	SS	SR + MR 1 colony	SR + M 1 colony
		7	"	"	SS	SR + MR +	SR + M +
		8	"	"	SS	SR + MR +	SR + M +
1927 1/3	37	9	"	"	SS	SR + + + + +	SR + + M 1 colony
		10	"	"	SS	SR + + + + +	SR + + E + M 1 colony
		11	"	"	SS	SR + + + + +	SR + +
		13	"	"	SS	SR + + + + +	SR + E + M 1 colony
23		9	"	"	SS	0	0
		10	"	"	SS	0	0
		11	"	"	SS	0	0
		13	"	"	SS	0	0

SS = typical smooth colony; SR = small, round, blue colony; E = small, wart-like, "eaten" colony; Ring = smooth colony surrounded by mucoid ring; MR = mucoid colony; MS = mucoid colony.

10 per cent bacteriophage agar. One set was incubated at 37°, the other left at room temperature, 23°. Single cell cultures of Strains 1, 2, 3, 4, 5, 6, and 7, and single colony Strains 8, 9, 10, 11, and 13 were used. These stock strains have been described in the previous experiment.

The results of these tests are given in Table IV. All strains grown on plain agar at 37° gave rise to smooth colonies; at 23° Strain 2 formed mucoid colonies (see Experiment 2). Subcultures from these smooth and mucoid colonies proved susceptible to lysis in broth containing bacteriophage and grew poorly or not at all on 10 percent bacteriophage agar plates. Hence these and all other typical smooth colonies have been designated smooth-susceptible (SS) (Fig. 1), and similar mucoid variants, mucoid-susceptible (MS). On the 1 per cent bacteriophage agar plates there appeared distinctly less growth and a different type of colony. Instead of the typical smooth-susceptible colonies, there arose small, round, bluish, smooth forms. Some of these were wart-like, with irregular, "eaten" edges and raised centers. Organisms from these colonies resisted lysis; hence the colonies were designated smooth-resistant (SR) (Fig. 2). On plates left at room temperature—23°—mucoid- and smooth-resistant colonies with a mucoid ring occurred, in addition to the smooth-resistant forms. These mucoid colonies likewise proved phage-resistant and hence were designated mucoid-resistant (MR) (Fig. 3). Growth on the 10 per cent phage agar plates was still more scanty. On plates incubated at 37° a few scattered smooth-resistant colonies were seen, with an occasional mucoid or ring form. On plates left at 23° there was less growth. Some remained sterile.

Further experiments with other strains gave similar results. In one series, four single cell strains were passed eight times on plain and bacteriophage agar. Each transfer showed the same types of colony variants as described above. From two guinea pig strains, however, rough colonies occurred regularly on phage agar plates. These resisted lysis and were designated rough-resistant (RR) (Fig. 4). Rough-resistant colonies were also obtained with great regularity by transferring smooth-resistant colonies to plain agar for two or three passages. In these two instances, a fluid medium did not seem to be necessary for the production of rough colonies.

TABLE V.
Lytic Action of Bacteriophage on Smooth Type B. enteritidis.
Broth Cultures.

Incubation hrs.	Culture dilution 1:10					Culture dilution 1:100					Culture dilution 1:10,000					Culture dilution 1:100,000										
	Control	Phage 1:10	G.	1:100	G.	1:100,000	G.	1:100,000	G.	1:10,000,000	Control	Phage 1:10	G.	1:100	G.	1:100,000	G.	1:10,000,000	Control	Phage 1:10	G.	1:100	G.	1:100,000	G.	1:10,000,000
	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.
3	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	+	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	+	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	+	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
72	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0

Asparagine—Salt Cultures.

3	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	+	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	+	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	+	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	+	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72	+	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

G. = growth; 0 = no growth; R = granular growth.

1, +, +1, ++, +++ = variations in turbidity from minimum to maximum growth (1,000,000,000 per cc.).

TABLE VI.

Colony Types on Agar Transfers from Broth and Asparagine Cultures Containing Bacteriophage.
Broth Cultures.

Incubation hr.	Culture dilution 1:10					Culture dilution 1:100					Culture dilution 1:10,000					Culture dilution 1:100,000				
	Control		Phage 1:10	1:100	1:100,000	Control		Phage 1:10	1:100	1:100,000	Control		Phage 1:10	1:100	1:100,000	Control		Phage 1:10	1:100	1:100,000
	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
7	S + + + + +	S + + + + +	0	+	0	S + + + + +	S + + + + +	0	0	+	S + + + + +	S + + + + +	0	0	+	S + + + + +	S + + + + +	0	0	+
10	S + + + + +	S + + + + +	S + + + + +	R +	0	S + + + + +	S + + + + +	R +	S + + + + +	E + + + + +	0	S + + + + +	S + + + + +	0	R + + + + +	+	S + + + + +	R + + + + +	0	+
18	S + + + + +	S + + + + +	S + + + + +	R + +	S + + + + +	S + + + + +	S + + + + +	M +	S + + + + +	E + + + + +	R + +	S + + + + +	S + + + + +	0	R + + + + +	M + +	S + + + + +	R + + + + +	0	+
22	S + + + + +	S + + + + +	S + + + + +	R + +	S + + + + +	S + + + + +	S + + + + +	R + +	S + + + + +	S + + + + +	M + +	S + + + + +	S + + + + +	0	R + + + + +	M + +	S + + + + +	R + + + + +	0	+

Asparagine Cultures.

Incubation hr.	Control		Phage 1:10		1:100		1:100,000		Control		Phage 1:10		1:100		1:100,000		1:10,000,000		Control		Phage 1:10		1:100,000	
	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
7	S + + + + +	S + + + + +	0	+	0	+	0	S + + + + +	S + + + + +	0	S + + + + +	+	0	+	+	+	+	+	+	+	+	+	+	+
10	S + + + + +	S + + + + +	S + + + + +	R +	S + + + + +	R +	S + + + + +	R +	S + + + + +	R +	S + + + + +	R +	S + + + + +	R +	S + + + + +	R +	S + + + + +	R +	S + + + + +	S + + + + +	0	+	+	+
18	S + + + + +	S + + + + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	S + + + + +	0	+	+	+
22	S + + + + +	S + + + + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	R + +	S + + + + +	S + + + + +	0	+	+	+

C = colonies; S = smooth colony (possibly includes SR colonies); M = mucoid-resistant colony; R = rough-resistant colony; E = smooth phage-colony; - = no agar transfer.

0 = no colonies; + = 1 - 10 colonies; ++ = 11 - 20 colonies; +++ = 21 - 50 colonies; ++++ = 50 + colonies.

Experiment 6.—This experiment was planned to test the effect of growth in bacteriophage-containing broth on colony formation.

Two fluid media were used, beef infusion broth, pH 7.4, and a modification of Uchinsky's asparagine solution.⁴ The test was run in duplicate in each medium. The single cell Strain 3 was employed. 17 hour broth and asparagine cultures from the stock agar slant were diluted in the corresponding media to make final bacterial concentrations of 5×10^{-1} , 10^{-2} , 10^{-4} , and 10^{-5} . Five cultures of each dilution were made; one served as a control and the other four received bacteriophage in dilutions of 6×10^{-1} , 10^{-2} , 10^{-5} , and 10^{-7} . The tubes were incubated at 37°. At short intervals thereafter the amount of growth was estimated in terms of turbidity and a loop of the culture was transferred to 15 cm., plain agar plates. These in turn were incubated and examined at 24 and 48 hours for number and types of colonies present. The findings are summarized in Tables V and VI.

In the broth series the control tube, inoculated with about 100,000,000 organisms, dilution 1:10, showed growth at 4 hours and maximum turbidity at 12, while tubes containing bacteriophage showed no definite growth for 12 hours, and did not reach a maximum until 27 hours. Tubes receiving an inoculation of 10,000,000 organisms, dilution 1:100, appeared quite similar, except that growth began later in all tubes. The control tube containing 100,000 organisms, dilution 1:10,000, showed abundant growth at 12 hours; similar tubes with high concentration of bacteriophage, 6×10^{-1} and 10^{-2} , remained sterile, while those in which bacteriophage was present in smaller amounts, 6×10^{-5} and 10^{-7} , showed growth at 20 hours and maximum turbidity at 30 hours. The control tube inoculated with 10,000 organisms, dilution 1:100,000 showed growth at 12 hours, which became maximum at 24 hours; those receiving high concentrations of bacteriophage remained sterile, and those in which the amount was low were clear at 30 hours and of maximum turbidity at 48 and 55 hours.

Rough colonies appeared in greatest numbers on plates from tubes inoculated with high concentrations of bacteriophage. Mucoid-resistant colonies were found chiefly on plates from tubes inoculated

⁴ The composition of the synthetic medium was as follows: Asparagine, 0.4 per cent; ammonium lactate, 0.6 per cent; sodium phosphate, 0.2 per cent, and sodium chloride, 0.6 per cent in distilled water.

with small amounts of bacteriophage. No variants occurred on the plates from plain broth tubes. The asparagine media series gave similar results, except that growth was slower in all tubes and mucoid-

TABLE VII.

Passage of Variant Colony Forms on Bacteriophage-Containing Agar and Plain Agar.

Date	Pas- sage	Smooth-resistant strains. Passage on 10 per cent phage agar at 37°			Pas- sage	Mucoid-resistant strains. Passage on 10 per cent phage agar at 23°			Pas- sage	Rough-resistant strains. Passage on 10 per cent phage agar at 37°	
		No. 1	No. 3	No. 4		No. 1	No. 3	No. 4		No. 3	No. 4
1927											
2/14	1	SR	SR. MR	SR. MR	1	MR	MR	MR			
15	2	"	"	"	2	"	"	"			
17	3	"	"	"	3	"	"	"			
19	4	"	"	"	4	"	"	"			
21	5	"	"	"	5	"	"	"	1	E. RR	E. RR
23	6	"	"	"	6	"	"	"	2	" "	" "
25	7	"	"	"	7	"	"	"	3	"	"
28	8	"	"	"	8	"	"	"	4	"	"
30									5	"	"
		Passage on plain agar 37°				Passage on plain agar 23°				Passage on plain agar 37°	
2/14	1	SS	SS	SS	1	MR	MR	MR			
15	2	"	" RR	"	2	"	"	"			
17	3	"	"	"	3	"	"	"			
19	4	"	" RR	"	4	"	"	"			
21	5	"	"	"	5	"	"	"	1	RR	RR
23	6	"	"	"	6	"	"	"	2	"	"
25	7	"	"	"	7	"	"	"	3	"	SS
28	8	"	"	"	8	"	"	"	4	SS	"
28	8	—		—	8	SS*	SS*	SS*			
30									5	"	"

SS = smooth-susceptible colony; SR = smooth-resistant colony; MR = mucoid-resistant colony; RR = rough-resistant colony; E = wart-like, "eaten" colony.

* Duplicate passage at 37°.

resistant colonies were not obtained. In four other similar experiments, however, both mucoid-resistant and rough-resistant colonies were recovered.

Briefly, then, bacteriophage added to nutrient media stimulates

the transformation of smooth colony types to mucoid and rough. Solid media and low temperature favor the mucoid forms; fluid media usually favors the development of rough types. These mucoid and rough colony forms differ from those obtained in plain media (Experiments 1, 2, 3) in that they resist the lytic action of bacteriophage.

The stability of these colony variants was then tested by growing them on plain and bacteriophage-containing media. The following experiment shows the result of passage on solid media with or without the addition of bacteriophage.

Experiment 7.—Cultures from the stock agar slant were streaked directly over the surfaces of plain and phage-containing agar plates. These were incubated at 37° and 23°C. Two single colonies of each variant type resulting were then selected and passed for a number of times on plain and bacteriophage-containing agar. Two well isolated colonies were chosen for each transfer. The results of these passages are shown in Table VII.

Three strains of smooth-resistant variants (SR) remained type-pure for eight passages on 10 per cent phage-containing agar, but on plain agar reverted almost immediately to the original smooth-susceptible colony form. The appearance of a few rough colonies on the second and fourth passages of Strain 3 is consistent with the results obtained in Experiment 4. Three mucoid-resistant variants (MR) remained constant for eight passages on 10 per cent phage agar at 23°, and on plain agar as well. But such variants grown on plain agar at 37° revert quickly to the smooth-susceptible form. Two rough-resistant variants passed on 10 per cent phage agar became small and wart-like; passed on plain agar, they reverted to the smooth form after two or three passages. Some rough variants, however, have remained constant for as many as fourteen passages before reverting to smooth.

Hence, the tests indicate that smooth-resistant, mucoid-resistant, and rough-resistant colony cultures, grown on bacteriophage-containing agar, tend to remain type-pure, but when placed on plain agar, become transformed to the original smooth type colony form.

Passage of variant forms in fluid media was carried out with a number of single cell strains from smooth-resistant, mucoid-resistant, and rough-resistant variant colonies. Each culture was transferred

from stock agar to plain broth and broth containing varying amounts of bacteriophage. After 24 hours incubation, a loop of plain broth culture was transferred to a fresh tube of broth, and a loop of bacteriophage-broth culture to a fresh tube of bacteriophage broth. At the same time subcultures were made on plain agar plates. These were incubated and examined at 24 and 48 hours.

When passed in plain broth for more than two or three transfers, single cell cultures from rough-resistant and mucoid-resistant colonies streaked on plain agar plates at 37° showed smooth-susceptible colonies. Smooth-resistant colony cultures reverted to smooth-susceptible after six or seven broth transfers. Bacteriophage added to the broth in a ratio of 10 per cent or more tended to inhibit the reversion phenomenon and maintain the variant cultures type-pure. Smooth-resistant strains remained relatively constant, mucoid-resistant forms remained so at 23° and became smooth-resistant at 37°; rough-resistant cultures usually showed some smooth reverted forms when grown at 37° and mucoid-resistant forms at 23°. Thus, in fluid culture, the variant colony strains were found to revert quickly to the original smooth-susceptible type, unless bacteriophage in large quantity were present and continually added to the cultures.

DISCUSSION AND SUMMARY.

The experiments described in this paper are part of a larger number which we have been carrying out for the past 2 years. Although they have not entirely fulfilled their purpose, which was to explain the so called "mutation," "dissociation," "Umwandlung," "transformation" process, they have served to formulate a helpful working hypothesis.

Certain difficulties inherent in this kind of study must be recognized. One is that colony formation is a property associated with growth on solid media and any procedure involving the use of fluid media introduces a change which is relatively uncontrolled. Another is that mucoid and rough colony forms are not the only variant types encountered; at best, they may be considered as being the most frequent. Finally, it is apparent to us that the findings reported in this paper with respect to enteritidis organisms do not correspond with those of other species of organisms, especially of the respiratory

group which we have studied (1, *b*). Possibly, however, they apply generally to the typhoid, paratyphoid-enteritidis species.

To summarize, it may be stated that the transformation process in the mouse typhoid enteritidis group is an easily reversible one, controlled in part, at least, by three factors, any of which may conceivably operate under natural conditions: (1) temperature, which, influences the appearance of mucoid forms; (2) fluidity of culture media, which tends to favor rough variants; and (3) bacteriophage, which stimulates the appearance of both variants. Since by manipulating these factors the transformation process may be incited at will in either direction, it is probably not genetic in nature.

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EXPLANATION OF PLATE 33.

- FIG. 1. Smooth-susceptible colonies of *B. enteritidis*.
FIG. 2. Smooth-resistant colonies of *B. enteritidis*.
FIG. 3. Mucoid-resistant colonies of *B. enteritidis*.
FIG. 4. Rough-resistant colonies of *B. enteritidis*.



STUDIES ON THE MODE OF SPREAD OF *B. ENTERITIDIS* MOUSE TYPHOID INFECTION.

III. STUDIES OF BACTERIAL CELLS TAKEN FROM SMOOTH, MUCOID, AND ROUGH COLONIES.

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The experiments described in the previous paper (1, *a*) indicate that in general the so called colony "mutation," "Umwandlung," "dissociation," or "transformation" process, as it occurs in mouse strains of *B. enteritidis*, is not of a genetic nature, but is a reversible phenomenon, influenced by at least three factors: temperature, fluidity of medium, and presence of specific bacteriophage. Further knowledge of the nature of the transformation process was sought by making a comparison of the bacterial cells contained in the smooth and the variant colonies.

Appearance of Individual Cells during Early States of Multiplication.

At the outset, single bacilli were examined during early multiplication in order to detect morphological differences in individual young cells taken from smooth, mucoid, and rough colonies.

Reimann's technique (2) was employed. 2 or 3 drops of melted agar were placed on a sterile, hollow ground slide and allowed to cool. Subsequently a small loop of a suspension of cells from a given colony, diluted in sterile 0.85 per cent salt, was placed on the surface of the agar. The dilution was so adjusted that a loop contained 10 to 20 bacterial cells. The preparation was then placed on a microscope stand and examined in a warm box with a dry objective at a magnification of 690 diameters. Aseptic precautions were maintained throughout.

Single cells from smooth-susceptible, smooth-resistant, mucoid-susceptible, mucoid-resistant, and rough-resistant colonies were fol-

lowed through the 2, 4, 8, 16, and 32 cell stages. Many strains were used, but no differences in cell morphology or behavior were detected. In so far as we could determine, young bacilli from the various colony types were indistinguishable.¹

TABLE I.

Growth of Smooth and Variant Strains of B. enteritidis in Broth.

Incubation time	Average bacterial count per cc.					
	1/3/26 Smooth- susceptible	1/3/26 Mucoid- susceptible	1/3/26 Rough- resistant	1/6/26 Smooth- resistant	1/6/26 Mucoid- resistant	1/6/26 Rough- resistant
<i>hrs.</i>						
0	12	9	5	11	14	11
1	21	18	8	8	18	12
2	65	32	22	31	30	45
3	216	88	127	178	245	191
4	1870	560	430	975	1190	1540
5	8600	3040	3473	6380	11,093	9800
6	52,533	13,000	10,000	35,400	59,600	75,533
7	380,000	140,000	290,000	288,700	443,000	582,000
8	2,850,000	390,000	595,000	1,260,000	3,347,000	4,300,000
9	16,033,333	4,250,000	2,000,000	9,133,333	18,900,000	15,800,000
10	63,533,333	19,270,000	216,000,000	43,300,000	92,533,333	135,933,333
24-26	940,000,000	820,000,000	920,000,000	1,085,000,000	1,470,000,000	1,370,000,000
<i>days</i>						
103-110	2,113,000	48,200	3,520,000	1,920,000	0	334,000
286	—	—	—	0	0	0

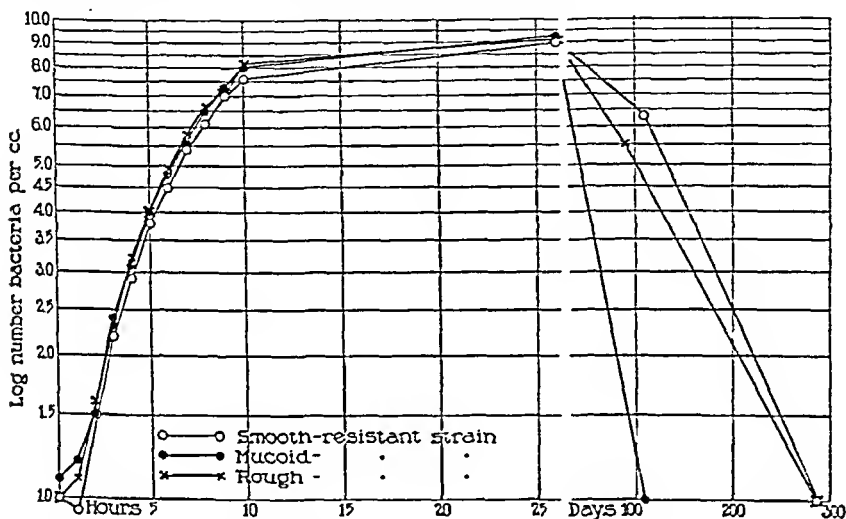
Dark-field illumination and staining methods likewise failed to exhibit any distinguishing characteristics.

¹A somewhat similar study of smooth and rough cells has been made by Nutt (3). She found that "rough cultures showed the typical bending type of division, producing at the end of the day, irregular masses with projecting angles. In the smooth cultures, the sliding divisions gave rise to the smooth, even masses typical of the smooth colony." Apparently, the emulsions used for seeding in these observations were not washed, but contained the metabolic products which accumulate during cell growth. It is not impossible, therefore, that unwashed cells do, at the outset, behave differently, whereas washed cells behave similarly until metabolic substances accumulate.

Rate of Multiplication of Cells from Smooth and Variant Colonies.

Tests were then made of the growth rates of single cell bacterial cultures obtained from smooth and variant colonies.

Experiment 1.—Variant colonies of Strain 3 were obtained from a single cell smooth-susceptible colony strain by methods outlined in the preceding paper (1, a). From these colonies, single cell cultures were procured, according to Reimann's technique (2). These variant strains were transferred to stock agar slants and tested carefully for type purity before use.



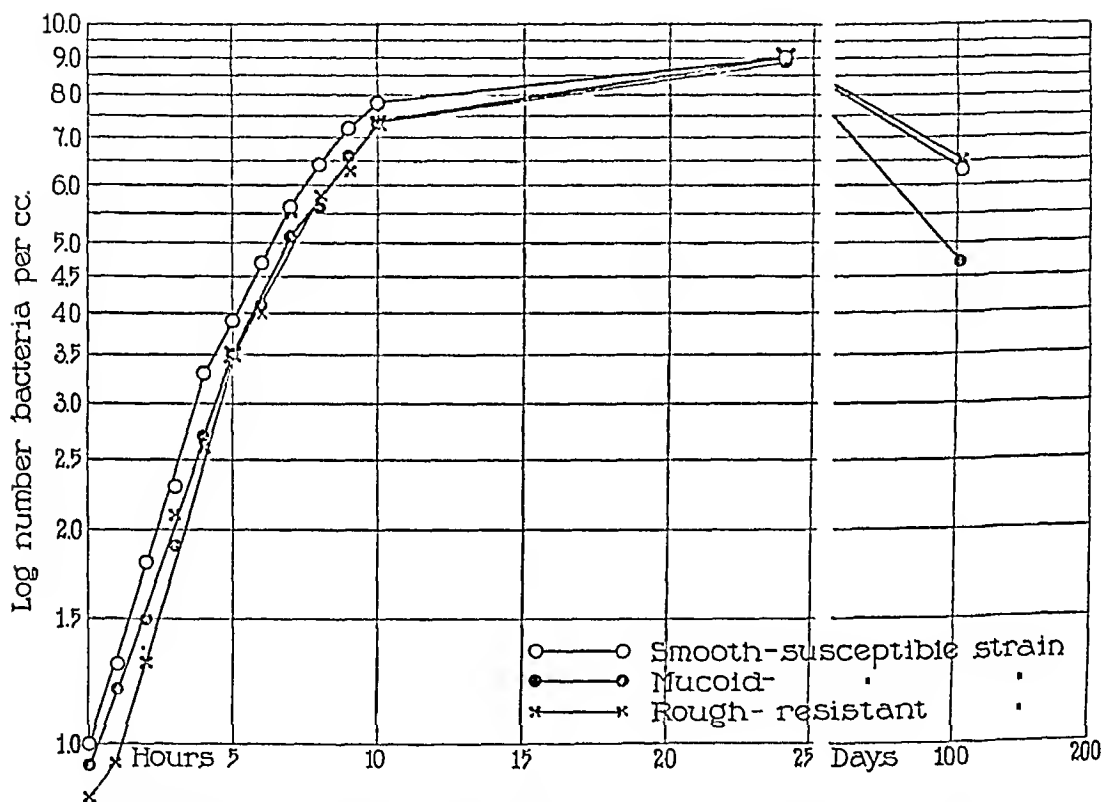
TEXT-FIG. 1. Growth of single cell cultures of *B. emeritidis* in broth. Smooth-resistant, mucoid-resistant, and rough-resistant strains.

18 hour broth cultures from the stock single cell smooth and variant strains were diluted in broth to 1/1,000,000. 1.5 cc. of this dilution was added to 150 cc. of broth in Erlenmeyer flasks, at 37°. Counts were made immediately, and thereafter at hourly intervals, for 10 hours. Further counts were made at 24 hours, and 100 and 280 days. The plating method was employed for counting, and care taken to insure the type purity of each strain. The results of this experiment are shown in Table I and Text-figs. 1 and 2.²

² These observations were made by Dr. I. W. Pritchett.

The figures (Table I) and curves (Text-figs. 1 and 2) indicate that the growth rates of single cell strains from smooth, mucoid, and rough type colonies are essentially identical.

Thus it appears that in morphology, gross behavior, and growth rate, young cells, 1 to 10 hours old, from any of the several colony



TEXT-FIG. 2. Growth of single cell cultures of *B. enteritidis* in broth. Smooth-susceptible, mucoid-susceptible, and rough-resistant strains.

types studied, are indistinguishable. The next tests were planned to compare certain fundamental properties of mature cells 18 to 24 hours old, taken from different type colonies.

Rate of Migration in an Electric Field.

Cataphoresis measurements were undertaken to compare the potentials of single cell bacilli obtained from smooth and variant colonies.

The Northrop type of cataphoresis cell was employed and standardized according to his methods (4). Readings were taken at 3/16, 7/16, and 11/16 of the cell depth, using both directions of current. The current was regulated to give a potential difference in the cell of 5 volts per cm. From the formula,

$$\frac{\mu (83.33)}{\frac{\text{No. of sec.}}{5}} \times 14,$$

the potential in millivolts was calculated. 18 hour growths of single cell cultures were used, and in the tests reported, washed four times in distilled water and suspended in Northrop's buffer (5).

TABLE II.

Rate of Migration of Cultures from Smooth and Variant Colonies.

Test	Source of culture									
	SS		SR		MS		MR		RR	
	sec.	mv.	sec.	mv.	sec.	mv.	sec.	mv.	sec.	mv.
1	5.2	45.2	—	—	5.1	45.7	5.1	45.7	—	—
							5.0	46.7		
2	5.6	41.8	5.7	40.9	—	—	—	—	5.9	39.5

SS = single cell culture from smooth-susceptible colony; SR = single cell culture from smooth-resistant colony; MS = single cell culture from mucoid-susceptible colony; MR = single cell culture from mucoid-resistant colony; RR = single cell culture from rough-resistant colony.

Table II shows the results of two tests. The migration rate and calculated potential of cells from different colonies were found to be similar. Other tests on unwashed cells and washed cells suspended in distilled water, 0.85 per cent NaCl, or 1 per cent phosphate affected the migration rate in the usual manner (6), but brought out no differences among the various cultures employed.

Oxygen Absorption (Determinations by Dr. Hawkins).

An attempt was made to compare the respiration rate of single cell cultures from smooth and variant colonies by measuring the amount of oxygen absorbed by each under conditions of equilibrium.

Technique.—Single cell cultures from smooth and variant colonies were grown in broth 18 to 24 hours, and washed four times in Ringer's solution containing 0.025 mols of sodium bicarbonate per liter. Final suspensions were then made in Ringer-bicarbonate solution in such a way as to contain relatively the same number of bacilli per cc. Bacterial counts were made before and after the tests to determine the actual numbers of living organisms in each suspension. The cultures were then given to Dr. James Hawkins who determined the amount of oxygen absorbed by each culture.³

TABLE III.

Type	No. of bacteria in millions per cc. of suspension	C.mm. of O ₂ used per hr. per 100,000,000 of bacteria
SS	71	2.3
	45	2.3
	200	1.6
SR	55	2.7
	426	1.7
	174	2.6
RR	80	1.9
	727	1.0
	125	1.8
MR	278	1.3
	218	2.6
	245	2.8
MS	310	1.2
	400	1.3
	140	2.7

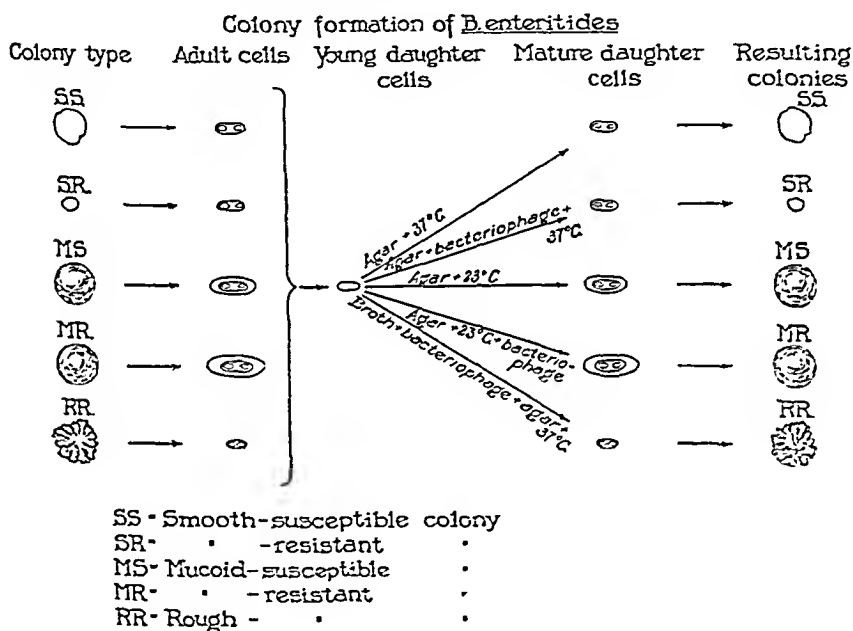
SS = cells from smooth-susceptible colony; SR = cells from smooth-resistant colony; MS = cells from mucoid-susceptible colony; MR = cells from mucoid-resistant colony; RR = cells from rough-resistant colony.

The hydrogen ion concentration was adjusted to pH 7.6 by bubbling through the suspension a mixture of 5 per cent carbon dioxide and 95 per cent oxygen. 8 cc.

³Full descriptions of Dr. Hawkin's technique are to be found in the following papers: Warburg, O., and Minami, S., *Klin. Woch.*, 1923, ii, 776. Warburg, O., Negelein, E., and Posener, K., *Klin. Woch.*, 1924, iii, 1062. Warburg, O., *Biochem. Z.*, 1923, cxlii, 317. Minami, S., *Biochem. Z.*, 1923, cxlii, 334. Warburg, O., *Biochem. Z.*, 1924, clii, 51. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309. Murphy, Jas. B., and Hawkins, J. A., *J. Gen. Physiol.*, 1925, viii, 115.

and 3 cc. portions of the suspension were measured accurately into a Warburg type of cell. 5 cc. of this same Ringer's solution, without bacteria, was placed in another cell to act as a control for change in temperature and barometric pressure. The cells were attached to Barcroft manometers, and after the residual air in the cells and manometers had been swept out by the oxygen-carbon dioxide mixture, they were sealed. The manometers were placed on a mechanical shaker with the cells immersed in a constant temperature bath at $37.5^\circ \pm 0.01^\circ\text{C}$. and shaken. The manometers, with the cells immersed in the constant temperature bath, were left for 15 minutes to allow them to come to equilibrium and were then read every 15 minutes.

The amount of oxygen consumed by the bacteria is determined in c.mm. per hour. The results are shown in Table III.



TEXT-FIG. 3. Diagram showing theory of colony formation of *B. enteritidis*.

These figures indicate that the oxygen absorption rate per unit of time per cell in suspensions from the typical smooth-susceptible colonies and their mucoid and rough variants is approximately the same. Hence, the rate of respiration, a fundamental cell property, may be considered uniform for the cells of the different type colonies.

The various observations thus far made were designed to bring out fundamental differences between bacilli from smooth and variant colonies. That they failed to do so, in that no differences in (1)

morphology, or (2) growth rate were noted in young cells, and no differences in (3) potential, or (4) in respiration rate in mature cells, does not prove that young bacilli from each of the several colony types are essentially the same. However, the tests do point in that direction, and hence confirm the theory stated in the preceding paper. Accordingly, one may consider colony morphology as the gross appearance of a population of adult microbes fundamentally similar but modified by certain environmental circumstances, rather than as the expression of any special genetic characters common and specific to each individual in the population. Such a view would predicate the genetic uniformity of young daughter cells derived from individuals of all the colony types, and would explain colony modifications as representative of acquired properties occurring during the lifetime of the individual bacilli, in response to certain definite environmental conditions (Text-fig. 3).

Staining Characteristics.

Notwithstanding this, cells from 18 to 24 hour growths do differ in certain properties according to the colony type from which they originated. One of these differences is in staining quality. Bacilli from the mucoid-susceptible or resistant colonies appear swollen and stain poorly. Capsules may be demonstrated. Bacilli from the rough-resistant colonies appear small and shrunken. The bacterial cytoplasm appears reduced in amount. These differences are seen very clearly when Wright's blood stain is used.

Differential Characteristics of Cells from "Susceptible" and "Resistant" Colonies.

Three other characteristics distinguish cells from *susceptible* and *resistant* colonies: namely, (1) their behavior in the presence of nutrient media containing bacteriophage; (2) their acid agglutination zone, and (3) their virulence.

Cell Behavior in the Presence of Bacteriophage.—Single cell cultures derived from smooth-, mucoid-, and rough-*susceptible* colonies grow poorly or not at all on agar containing 0.1 per cent or more specific bacteriophage, while similar implants from *resistant* colonies, smooth,

muroid, or rough, multiply luxuriantly and form type-specific resistant colonies. These differences have been discussed at length in the previous paper (1, *a*).

Differences in Acid Agglutination.—The second difference between *resistant* and *susceptible* colony cultures is that the former flocculate in acid buffers at pH 3.8 to 4.2, while the latter remain suspended.

Technique.—18 hour single cell cultures from the stock agar slant were tested for type purity and washed four times in distilled water. 1 cc. of a suitably diluted suspension was then added to 2 cc. of acid solutions of differing pH values. When Na acetate-acetic acid buffers were used, suspensions from the resistant colony types (smooth, muroid, and rough) flocculated at pH 2.5 to 4.0. In Na lactate-lactic acid buffers, they agglutinated at pH 3.0 to 3.8, and in dilutions of hydrochloric acid at pH 3.8 and 4.0 (7). Suspensions from susceptible colonies (smooth, muroid, or rough) did not flocculate at any acid dilution employed. LaCl_3 , however, from dilutions of 1/20 to 1/20,480 agglutinated all cultures alike.

Virulence of Single Cell Cultures from Smooth-Susceptible and Variant Colony Types.—Single cell cultures from bacteriophage-resistant colonies, smooth, muroid, and rough, proved markedly less virulent than cultures from the smooth-susceptible colony strains. The details of these experiments are contained in the following paper (1, *b*).

Action of Bacteriophage on Living, Non-Multiplying Bacterial Cells.

That the bacilli from various type colonies resemble one another closely in their fundamental characteristics and yet differ in acid agglutination, virulence, and resistance to bacteriophage, according to whether they come from *susceptible* or *resistant* colonies, suggests the possibility that the differences are due to an adsorption of bacteriophage on the surface of the *resistant* bacilli. Accordingly, this phenomenon, and its modifying effects would be considered as the result of acquired, not of inherited, alterations.

Experiments in another paper (1, *b*) support this view entirely. Furthermore, such a conclusion is in harmony with the latest studies on the mechanism of bacteriophage action. D'Hérelle (8), and all late investigators are of the opinion that the first step in bacteriophage action is a surface binding of phage and bacterial cell. Still another series of studies on the mechanism of bacterial agglutination are ex-

plained by considering flocculation as the result of a specific coating of bacteria by globulin (9).

We have sought further information as to (1) whether bacterial cells do adsorb bacteriophage and (2) whether *susceptible* cells in contact with bacteriophage under conditions where the possibility of multiplication is reduced to a minimum, do become altered to resemble cells from *resistant* colonies.

The technique of the tests was so arranged as to reduce as far as possible bacterial multiplication.

18 hour growths of single cell cultures in broth were washed four times in distilled water. 0.8 per cent NaCl gave similar results. The washed cells were then suspended in various buffer solutions, glycocoll (5), and 0.2 and 1 per cent phosphate. When distilled water or 0.8 per cent NaCl was used as the diluent, the pH of the systems dropped rapidly to 6 or 6.6, thus rendering the system unfavorable for the test. 0.2 per cent phosphate was finally employed as a standard.

The number of cells per cc. was estimated by comparison with standardized suspensions and checked by actual counts on dilution plates. The test suspensions with or without phage were then placed in the ice box at 4° from 4 to 144 hours. Preliminary tests and direct counts showed that very few cells were killed during the washing process, or during the first 24 hours in the ice box at 4°C., provided the cells were suspended in a concentration of not more than 10^9 per cc.

Specific bacteriophage was prepared in broth in the usual way and added to the suspensions in as small a volume as possible. Subsequent determination of the amount of bacteriophage remaining in the solution was obtained by throwing down the cells, removing the supernatant, heating it to 60° for 30 minutes, or preferably filtering it through an M Berkefeld candle. Dilutions of the filtrate were then made from unit volume to 10^{-9} in 5 cc. broth, and about 10,000 bacilli of an 18 hour single cell culture of the smooth-susceptible type strain added to each. The amount of growth in each tube at 37° was then recorded at a definite interval of 6 to 10 hours after inoculation.

Cells acted upon by bacteriophage at 4° were tested in several ways for comparison with cells from variant type colonies. After being removed from the ice box, the suspensions were centrifuged and the cells washed. Oxygen absorption rate, potential, and acid agglutination zones were determined, as well as virulence. The results of these several tests are given in Tables IV to VI.

Table IV shows the results of one of a number of such tests with single cell strains from the typical smooth-susceptible colonies. Under these conditions, 200,000,000 organisms per cc. removed all bacterio-

phage from the solutions of 1/1000 and 1/100 concentration. The 10 per cent solution of bacteriophage was not entirely exhausted; the

TABLE IV.

Action of Bacteriophage on Suspensions of Non-Multiplying Bacteria.

Organisms 2,000,000 per cc. 2/16/26	Count per cc. after 4°C., 18 hrs.	Amount of phage remaining in supernatant													Acid agglutination of bac- terial sediment																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
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supernatant, when filtered, definitely inhibited the growth of the test strain. Organisms in a solution containing phage in a dilution of 1/1000 and 1/100 did not agglutinate, while those in a 10 per cent

TABLE V.
Action of Bacteriophage on Suspensions of Non-Multiplying Living and Dead Bacteria.

Organisms 1,000,000 per cc. 2/28/26	Amount of bacteriophage remaining in supernatant										Acid agglutination of bacterial sediment																			
	Phage control	Dilution of supernatant										Na lactate-lactic acid buffers										N/10 HCl								
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	Control	Control	pH 2.4	2.7	3.0	3.3	3.5	3.8	4.1	4.5	4.7		Control	1	2	3	4	5	6	7
Living plus bouillon control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Living plus phase 1/10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Living plus phase 1/100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Living plus phase 1/10,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Living plus phase 1/1,000,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dead plus bouillon control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dead plus phase 1/10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dead plus phase 1/100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dead plus phase 1/10,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dead plus phase 1/1,000,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

0 = no agglutination; C = complete agglutination.

0 = no agglutination; C = complete agglutination.

— = no test; + = good growth at 8 hours; 0 = no growth at 8 hours.

solution flocculated at the same pH, 3.8 to 4.2, as do bacilli from phage-resistant variant colonies. A number of these tests under slightly differing conditions gave similar results.

Subsequently the ratio of organisms to quantity of bacteriophage was varied and the test repeated. The tests indicated that the ratio of number of organisms to amount of bacteriophage taken out of the solution is relatively constant and hence that in all probability, each bacillus takes up a certain and definite amount of the lytic substance.

Table V compares the activity of living and dead bacilli. A single cell culture of smooth-susceptible colony type bacilli was used, washed four times in distilled water, and divided into two portions. One was heated to 75°C. for 1 hour. Evidently dead bacilli take up bacteriophage from the solution, but to a less degree. Probably, however, there exists a definite quantitative relation as in the case of the living organisms. Furthermore, dead bacilli are not agglutinated in acid solution unless placed in contact with the bacteriophage. When so treated, however, they flocculate at pH 3.8 to 4.2, as do living cells of the same sort and cells from bacteriophage-resistant variant colonies.

Oxygen absorption and potential measurements were made on suspensions of washed cells in contact with bacteriophage at 4°C. They were found to take up the same amount of oxygen per unit of time and to migrate at the same rate in the electric field as control suspensions and cells from any of the various type colonies. Hence bacteriophage in contact with living smooth-susceptible cells in the resting state, as well as cells from variant colony types, did not affect these fundamental physiological properties.

Changes in virulence were determined by the usual procedures. Washed cultures in contact with bacteriophage at 4°C. were later washed again and given to mice intraperitoneally and *per os*. Similar suspensions without the addition of phage were used as controls. Not only were the former suspensions of low pathogenicity, but they failed to multiply in the body of the host and disappeared rapidly in a manner precisely similar to single cell cultures from phage-resistant variant colonies. That this was not due to lysis was made certain by the behavior of broth and agar plate transfers. The details of this experiment are described in the following paper (1, b).

The fact that cells from smooth-susceptible colonies may be treated with phage under conditions where the possibility of cell multiplication is reduced to a minimum and changed so that they behave in a way precisely similar to cells taken directly from the bacteriophage-

TABLE VI.

Action of Bacteriophage on Suspensions of Non-Multiplying Cells from Bacteriophage-Resistant Colony Types.

Organisms No. per cc.	Amount of bacteriophage remaining in supernatant											Acid agglutination of bacterial sediment Na lactate-lactic acid buffers									
	Phage control	Dilution of supernatant										Hydrogen ion concentration of buffers									
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Control	Control	2.1	2.7	3.0	3.3	3.5	3.8	4.1	4.7
1/100		0	0	0	0	0	0	0	0	+	+	+									
1/1000		0	0	0	0	0	0	0	0	+	+	+									
SR (115,000,000)													0	0	0	0	C	C	C	C	0
SR (115,000,000) plus phage 1/100	-	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	0
SR (115,000,000) plus phage 1/1000	-	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	0
MR (94,000,000)													0	0	0	0	C	C	C	C	0
MR (94,000,000) plus phage 1/100	-	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	0
MR (94,000,000) plus phage 1/1000	-	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	0
RR (120,000,000)													0	0	0	0	C	C	C	C	0
RR (120,000,000) plus phage 1/100	-	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	0
RR (120,000,000) plus phage 1/1000	-	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	0
- = no test; + = good growth at 8 hours; 0 no growth at 8 hours.													0 = no agglutination; C = complete agglutination.								

resistant variant colonies is considered by us as evidence that this type of change is purely an environmental phenomenon, and that the particular bacteriophage action is a surface adsorption process similar to that of agglutinins adhering to the surface of their specific bacterial cell. If this view is correct, one would expect cells from bac-

terioophage-resistant colonies to adsorb no further bacteriophage under similar conditions, and to remain unaltered.

Table VI summarizes the results of a test in which cell suspensions from smooth-, mucoid-, and rough-resistant colonies were treated with bacteriophage as in the previous tests. None of them removed any noticeable quantity of phage from the solutions, and none of them showed alterations in acid agglutination zone.

DISCUSSION.

The above experiments are a continuation of those reported in the preceding paper, the purpose of which has been to secure a better understanding of the mechanism of colony transformation and of the part it plays in determining the spread of mouse typhoid. The observations on colony variation, although somewhat irregular, indicate that type of colony depends upon environmental rather than hereditary factors. This point of view is supported by the present studies on bacterial cells taken directly from different type colonies, and by the fact that living bacteria from the typical smooth colonies, placed in contact with bacteriophage under conditions where cell multiplication was restrained, are altered so as to resemble the bacterial cells from the variant bacteriophage-resisting colonies.

To consider the transformation process to mucoid and rough variant colonies as one of environmental degradation, and the reversion to smooth type as mere accumulation of daughter cells in the absence of such environment, does not explain bacterial transformation in the hemorrhagic septicemic group of organisms. Here the process seems non-reversible, and cells from variant colonies are found to differ markedly in fundamental properties (1, c). We put forward the view stated merely as a working hypothesis, and as best explaining at the present time the facts at hand relating to the transformation of organisms of the mouse typhoid *B. enteritidis* group.

CONCLUSIONS.

1. During early stages of multiplication, single cells from smooth-, mucoid-, and rough-susceptible and variant colonies show no differences in morphology or growth rate.

2. Cells from 18 to 24 hour single cell cultures of these various colony types possess similar oxygen absorption and cataphoretic migratory rates. In staining property, the cells from mucoid colonies appear larger, and those from rough colonies smaller, than the typical cells from smooth-susceptible colonies.

3. Cells from bacteriophage-resistant colonies differ from those of bacteriophage-susceptible colonies in their ability to multiply luxuriantly in the presence of bacteriophage, and in their tendency to flocculate in acid solutions at pH 3.8 to 4.1, as well as in their low degree of virulence.

4. Cells from smooth bacteriophage-susceptible colonies in contact with bacteriophage under conditions where multiplication is restrained may be altered so as to resemble the cells from the bacteriophage-resistant colonies.

5. These facts furnish evidence that bacteriophage adheres to the surface of the bacterial cell and that the various cell changes and colony alterations are of an environmental rather than genetic nature.

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STUDIES ON THE MODE OF SPREAD OF *B. ENTERITIDIS* MOUSE TYPHOID INFECTION.

IV. THE RELATIVE VIRULENCE OF SMOOTH, MUCOID, AND ROUGH STRAINS.

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In another paper of the series, we pointed out that when bacteria from typical smooth colonies of mouse strains of *B. enteritidis* are grown under certain environmental conditions, they become transformed into mucoid and rough colony forms (1, *a*). Organisms from these "variant" colonies, especially the smooth, mucoid, and rough bacteriophage-resistant forms, were found to differ in certain respects from those of the original smooth strain (1, *b*). One of the distinguishing characters proved to be loss of virulence, a change which also was effected experimentally by placing smooth type bacteria in contact with bacteriophage. It is proposed now to describe the titrations of virulence carried out and to give details of two additional experiments which partly explain the virulence mechanism involved.

The question of the measurement of bacterial virulence has been referred to at length elsewhere (1, *c*). At this time it is sufficient to emphasize again the necessity of controlling the dosage variable by using known numbers of bacilli, and the host factor by inoculating a sufficient number of mice of one race, similar age and weight, and with no previous exposure to the specific infection. The bacteria should be introduced by way of the normal portal of entry, and the mice should then be placed in separate cages in order to reduce the number of bacilli reingested. Under these conditions the reaction of an adequately large group of mice should be relatively constant, a definite number of individuals dying at a relatively constant rate, others recovering, and still others remaining apparently unaffected.

These effects may be shown graphically by plotting total mortality per cent against time.

The mice employed in the titrations recorded below came from the Rockefeller Institute breeding room. They consisted of an albino strain, inbred for 15 years. At the time of inoculation each animal was 12 to 14 weeks old, and weighed 18 to 20 gm. They are believed to have been unexposed to *B. enteritidis* mouse typhoid, although a very occasional carrier (less than 1 per 1000) has been encountered from time to time among the stock animals (1, d). After inoculation by stomach tube, each animal was placed in a separate glass jar in a special room cared for by an experienced attendant.

TABLE I.

Relative Virulence of Smooth, Mucoïd, and Rough Strains of B. enteritidis.
Experiment 1.

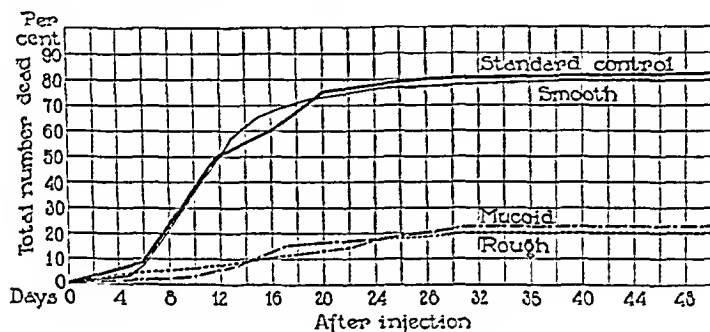
No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures; colony types	Results of fecal cultures			
		No.	Per cent	No.	Per cent		9th day		31st day	
							No. positive	Per cent and type of colonies	No. positive	Per cent and type of colonies
27	SS	22	82	22	82	Smooth 22; mucoid 2	3 (23%)	Smooth 2; mucoid 2	0	0
26	MR	6	23	6	23	Smooth 5; mucoid 5	5 (21%)	Mucoid 5	1 (5%)	Mucoid 1
27	RR	6	22	5	18.5	Smooth 2; rough 5	5 (20%)	Rough 5	2 (9.1%)	Rough 2

SS = culture from smooth-susceptible colony; MR = culture from mucoïd-resistant colony; RR = culture from rough-resistant colony.

Experiment 1.—The first test was made with the smooth type stock strain No. 3, obtained in 1922 from a case of spontaneous mouse typhoid (1, a, b). Its virulence was determined carefully in 1923 and found to be relatively constant and high (1, c). Since that time it has been kept in the ice box on an agar slant and transferred at irregular intervals. During October, 1925, the culture was treated with bacteriophage. This, when added to broth or agar cultures of the bacilli, led to the appearance of typical smooth, mucoïd, and rough colony variants which were resistant to lysis. The desired variant colonies were transferred to stock agar slants and identified and examined for type purity with a minimum number of subcultures.

Transfers from the stock agar cultures of the original smooth type and the

mucoïd and rough variants were made to broth and incubated for 17 hours at 37°. The following day suitable dilutions were made, plates were poured for counting colonies, and $\frac{1}{2}$ cc. of the 1-100 broth dilution of each culture was given *per os* to three series of mice. Each of twenty-seven mice received about 5,000,000 of the original smooth type bacilli; twenty-six mice, about 7,500,000 mucoïd variant bacilli, and twenty-seven mice 10,000,000 of the rough type. Each culture employed was found to be type-pure. Mice found dead from day to day during the experiment were autopsied and cultured from heart's blood, spleen, and intestine. The types and relative numbers of organisms were determined. Fecal cultures on all mice 3 days before inoculation were negative. 11 and 31 days after inoculation the surviving mice were cultured to determine the presence of *B. enteritidis* in their feces.



TEXT-FIG. 1. Relative mortality of mice given smooth, mucoïd, and rough strains of *B. enteritidis*. Experiment 1.

The protocols of this experiment are summarized in Table I and the specific mortality figures plotted in Text-fig. 1. 82 per cent of the mice receiving organisms of the original smooth type died, all showing pure cultures of smooth colonies in heart's blood, spleen, and intestine, and 9.1 per cent showing besides a few mucoïd forms. The mortality rate (82 per cent) corresponded almost exactly to that observed with the same strain 2 years previously (1, c). No mouse typhoid bacilli were obtained from the stool cultures of the survivors. 23 per cent of the mice receiving the mucoïd strain died. At autopsy one showed the mucoïd type only; four mucoïd and smooth types, and one the smooth type alone. Five of the survivors showed mucoïd types in their feces 11 days after injection, and only one at 31 days. From the remainder no mouse typhoid bacilli were obtained. 22 per cent

of the rough culture group died, and at autopsy one yielded no mouse typhoid bacilli from heart's blood, spleen, and intestine cultures, three rough forms only, and two both rough and smooth forms. Of the survivors five showed rough forms 11 days after injection, and one at the end of the 31 day period.

From the results of this virulence titration we conclude that the smooth type strain No. 3 of *B. enteritidis* is of relatively high pathogenicity, with exactly the same virulence as 2 years ago, and that its mucoid and rough variants are markedly less virulent. In the tissue of the mouse the smooth culture remained type-pure and virulent, except for the presence of an occasional mucoid colony,¹ while in many instances the mucoid and rough variants tended to revert quickly to the original smooth form.

2 weeks after the first titration a second was made, in order to compare the virulence of reverted and original smooth cultures.

Experiment 2.—Four strains were used—that designated smooth-susceptible No. 1, came from the original stock strain No. 3 and served as the control; smooth-susceptible Nos. 2 and 3 were obtained from smooth colonies found on autopsy plate cultures from two mice in Experiment 1 of the group inoculated with the mucoid culture; and smooth-susceptible No. 4, from a reverted smooth colony obtained from a plating of the mucoid culture used in Experiment 1, after six daily passages in broth.

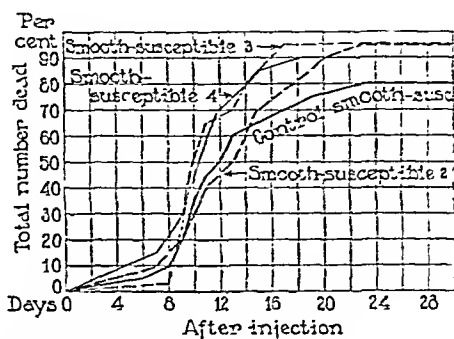
The technique employed for the inoculations was similar to that used in Experiment 1. Twenty mice received 5,000,000 bacilli of the original smooth-susceptible culture No. 1; twenty received 5,000,000 of the reverted smooth-susceptible No. 2 forms; twenty were given 4,500,000 of smooth-susceptible No. 3, and twenty 7,500,000 of smooth-susceptible No. 4 cultures. Each culture was tested carefully for type purity and specific characteristics. As far as could be determined, they were identical.

The specific mortality for each group is plotted in Text-fig. 2. The close similarity in amount and rate of deaths in the various groups indicates that the virulence of the different reverted smooth type cultures was the same as that of the original strain.

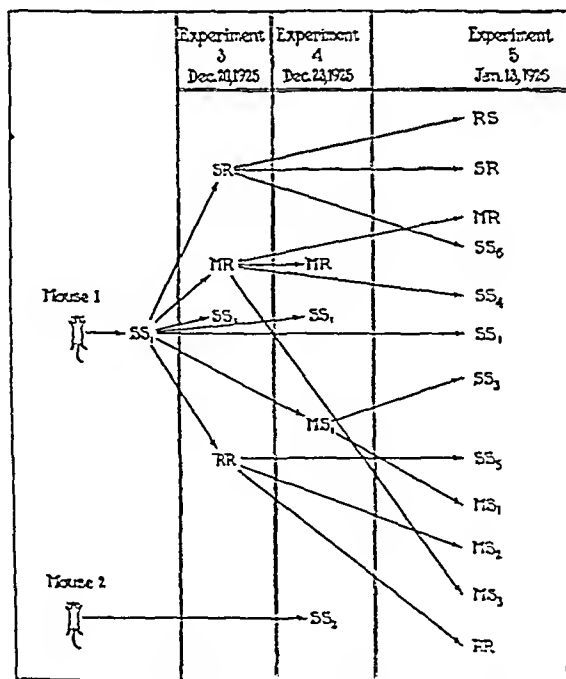
A further titration was made to test the virulence of recently isolated single cell cultures of smooth and variant strains of *B. enteritidis*.

¹ Plates were examined at 24, 48, and 72 hours. The presence of mucoid colonies was attributed to the fact that the plates had been left at 23° after 24 hours incubation at 37° (1, a).

Experiment 3.—A mouse from one of the experimental epidemic populations referred to above (1, d) was found dead on December 5, 1925. At autopsy, smooth type colonies of *B. enteritidis* were obtained in pure culture from heart's



TEXT-FIG. 2. Relative mortality of mice given smooth and "reverted" smooth strains of *B. enteritidis*. Experiment 2.



TEXT-FIG. 3. Diagram showing source of cultures used in Experiments 3, 4, and 5.

blood, spleen, and intestines. A single cell strain was procured from the heart's blood culture by Reimann's technique (2). A high titre bacteriophage was then obtained by daily passage and filtration of this single cell smooth type strain. This bacteriophage, added to young broth cultures of the single cell smooth type strain, gave rise to mucoid and rough variants resisting lysis. From these variant colonies single cell cultures were obtained, identified serologically, and tested for type purity.

17 hour broth transfers from the stock agar slants of these various single cell cultures (Text-fig. 3) were suitably diluted in broth, plated, and administered by stomach tube in $\frac{1}{2}$ cc. volume to four groups of mice. Each of twenty mice received 5,000,000 of the original smooth-susceptible strain; twenty were given 9,600,000 of the smooth-resistant variant; twenty 5,500,000 of the mucoid-resistant variant, and twenty 5,000,000 of the rough-resistant form. The animals were then placed in separate jars and treated precisely as in Experiments 1 and 2. On the following day the colonies on the dilution plates were examined carefully for type purity and identified serologically. All proved type-specific.

TABLE II.

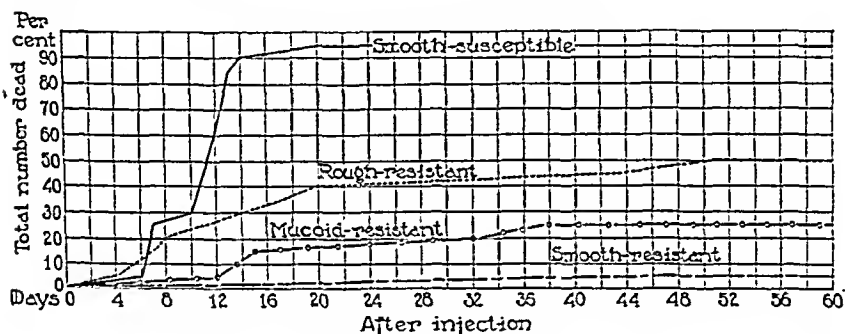
Relative Virulence of Smooth, Mucoid, and Rough Strains of B. enteritidis.
Experiment 3.

No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures: colony types
		No.	Per cent	No.	Per cent	
20	SS	19	95	19	100	Smooth 19; mucoid 8
20	SR	8	40	1	5	Mucoid 1
20	MR	15	75	5	25	" 5
20	RR	18	90	9	45	Rough 8; smooth 2

The results are summarized in Table II and specific mortality rates are plotted in Text-fig. 4. 95 per cent of the mice receiving the original smooth-susceptible type died. At autopsy smooth colonies were obtained from each animal and in addition mucoid forms from eight. Eight mice receiving the smooth-resistant variant succumbed; from one a mucoid form was recovered; from the remaining seven no mouse typhoid bacilli were obtained. 75 per cent of the mice given the mucoid-resistant culture died. Of these only 25 per cent showed *B. enteritidis*. Mucoid forms alone were present. 90 per cent of the rough series succumbed—only 45 per cent harbored the specific organisms. From 35 per cent of these, rough colonies alone were cultured; from one, both smooth and rough, and from one other smooth

types alone. Thus, two discrepancies were encountered in this experiment: first, the appearance of mucoid colonies in autopsy cultures of mice which had received smooth forms, and second, the death of a considerable number of mice giving negative autopsy cultures. The first peculiarity is explained by the fact that the autopsy plates were often left at room temperature after 24 hours incubation; the second we are not able to explain at the present time. It is interesting to note, however, that nearly all mice giving negative cultures died within the first 6 days, a period below the usual incubation time.

If specific deaths are plotted with reference to time, as in Text-fig. 3, the results appear quite consistent with those of the first two experiments, and indicate that the recently isolated single cell, smooth-



TEXT-FIG. 4. Relative mortality of mice given smooth, mucoid, and rough strains of *B. enteritidis*. Experiment 3.

susceptible culture is of high virulence, as in the case of those previously titrated, and that the single cell variants—smooth-, rough-, and mucoid-resistants—are relatively of low pathogenicity. Finally, there is further corroborative evidence that in the animal tissues the variant types (in this experiment, the rough form) revert to the smooth-susceptible type.

On December 23, 3 days after the last series was inoculated, a fourth titration of the same and other variant cultures was carried out.

Experiment 4.—The following strains were employed (see Text-fig. 3): SS 1, the original single cell, smooth-susceptible strain used in Experiment 3; SS 2, a single cell, smooth-susceptible culture similar in every respect to SS 1, obtained at the same time from another mouse in the epidemic series; MR 1, the same

single cell, mucoid-resistant variant from SS 1 used in Experiment 3, and MS 1, a single cell, mucoid-susceptible variant, obtained from an agar plate culture of the original SS 1, after three daily broth passages. Each culture was given to twenty mice—7,600,000 to each mouse in the SS 1 group; 5,900,000 to each in the SS 2 group, and 5,000,000 to each in the MS 1 and MR 1 groups. Tests for identification and type purity observations and autopsies were made precisely as in the previous experiments. All colonies on the dilution plates were type-pure and specific. The results are shown in Table III and Text-fig. 5.

90 per cent of the mice receiving the original SS 1 culture died, and showed at autopsy smooth-susceptible colonies in the heart's blood and spleen culture plates. In two cases besides, a few mucoid colonies were present. 95 per cent of the SS 2 series died: 85 per cent with

TABLE III.
Relative Virulence of Smooth and Mucoid Strains of B. enteritidis.
Experiment 4.

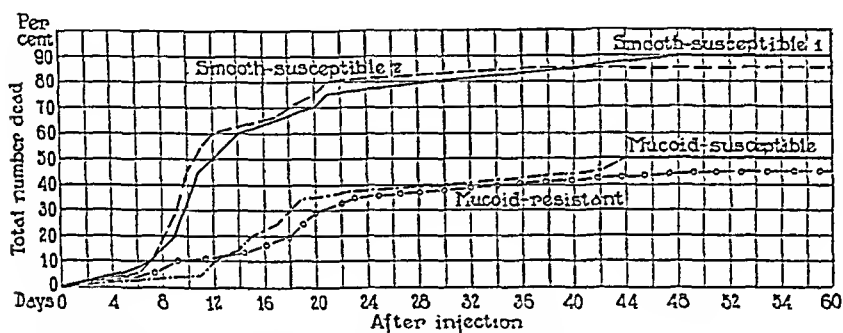
No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures: colony types
		No.	Per cent	No.	Per cent	
20	SS-1	18	90	18	90	Smooth 18; mucoid 2
20	SS-2	19	95	17	85	" 17
20	MS	14	70	10	50	Mucoid 10
20	MR	11	55	9	45	" 9

smooth-susceptible colonies in heart's blood and spleen cultures, and 10 per cent with no typhoid organisms whatsoever. 70 per cent of the MS series died; from 50 per cent type-specific organisms were recovered; from four, no mouse typhoid bacilli. 55 per cent of the MR series succumbed; 40 per cent with mucoid-resistant colonies in the autopsy cultures, one mouse with mucoid-susceptible forms, and two with no typhoid colonies. From a comparison of the curves in Text-fig. 4, which include only mice dying with positive typhoid cultures, it is evident that the two single cell, smooth-susceptible strains are of similar high pathogenicity, and that the single cell, mucoid-susceptible and mucoid-resistant variants are of comparatively low virulence. Little evidence of type reversion *in vivo* was obtained in this experiment.

A final titration was made with the original single cell, smooth-

susceptible Strain SS 1, used in Experiments 3 and 4, together with several of its variants and reverted forms.

Experiment 5.—The cultures were designated as follows (see Text-fig. 3): SS 1, MS 1, MR 1, SR 1, and RR 1, used in Experiments 3 and 4, and SS 3, SS 4, SS 5, SS 6, MS 2, MS 3, and RS 1, reverted forms derived from the above named cultures by repeated broth passage and plating. SS 3, a reverted, single cell, smooth-susceptible culture came from the mucoid-susceptible variant (MS 1); SS 4, a single colony, smooth-susceptible culture from the mucoid-resistant MR strain; SS 5, a single cell, smooth-susceptible culture from the rough-resistant RR variant, and SS 6, a single colony, smooth-susceptible culture from the smooth-resistant (SR) strain. MS 2, a single cell, mucoid-susceptible culture, came from the rough-resistant RR strain; MS 3, a single colony, mucoid-susceptible



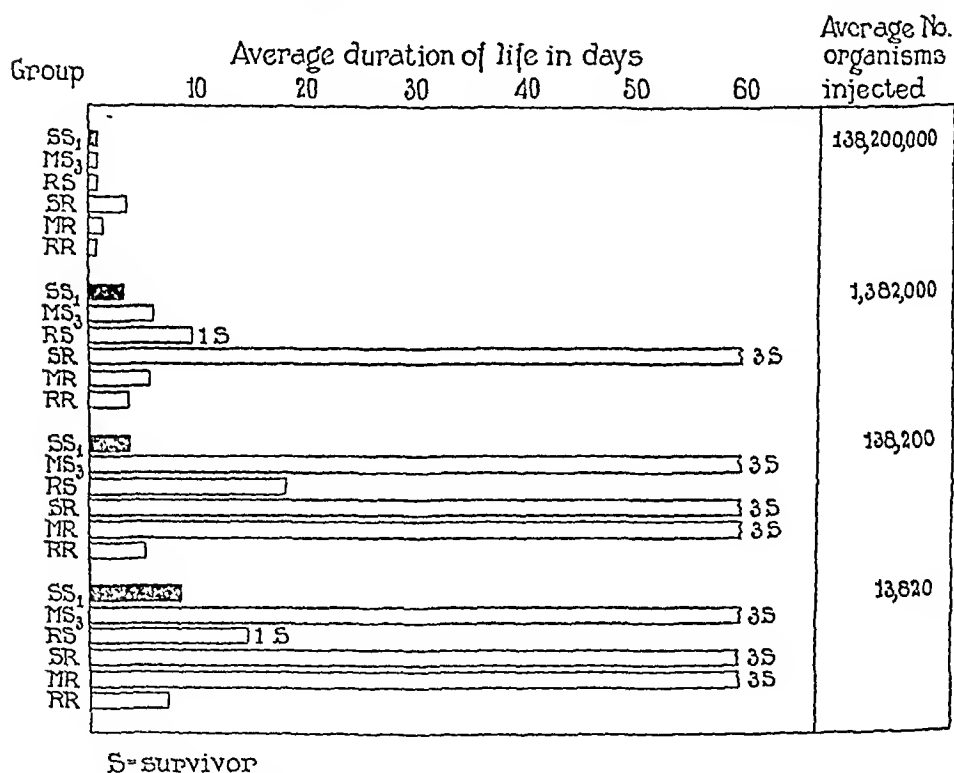
TEXT-FIG. 5. Relative mortality of mice given smooth and mucoid strains of *B. enteritidis*. Experiment 4.

culture, from the mucoid-resistant MR strain, and RS, a single cell, rough-susceptible culture, from the smooth-resistant SR strain.

On January 13, 1926, 17 hour broth cultures from the stock agar slants were diluted in broth, plated, and given *per os* to twelve series of mice. Each series consisted of twenty animals obtained from the same source, and cared for in the same way as those used in the previous experiments. The dosage per mouse of each culture was as follows: SS 1, 9,000,000; SS 3, 7,500,000; SS 4, 9,000,000; SS 5, 9,000,000; SS 6, 8,875,000; MS 1, 7,250,000; MS 2, 14,000,000; MS 3, 5,000,000; RS 1, 4,250,000; SR 1, 7,625,000; MR 1, 6,750,000; and RR 1, 8,885,000. The SS 1, MS 3, SR 1, MR 1, RR 1, and RS 1 strains were also injected intraperitoneally into similar mice. Each strain was administered to twelve mice. Three received a dilution of 10^{-1} ; three, 10^{-2} ; three, 10^{-3} ; and three, 10^{-4} . The actual dosage in numbers of bacilli was calculated from counts of the dilution plates.

On the following day the plates were tested for type specificity and purity. The mice placed in individual jars were observed for a period of 2 months, and those dying were autopsied and cultures were made from the spleen, heart's blood, and intestines. The types and relative numbers of *B. enteritidis* colonies were determined by inspection, sugar fermentation, agglutinability in specific sera, and lysis in bacteriophage-containing broth.

The dilution plates of each culture, after 24 hours incubation, showed type-specific and pure colonies. Text-fig. 6 shows the results



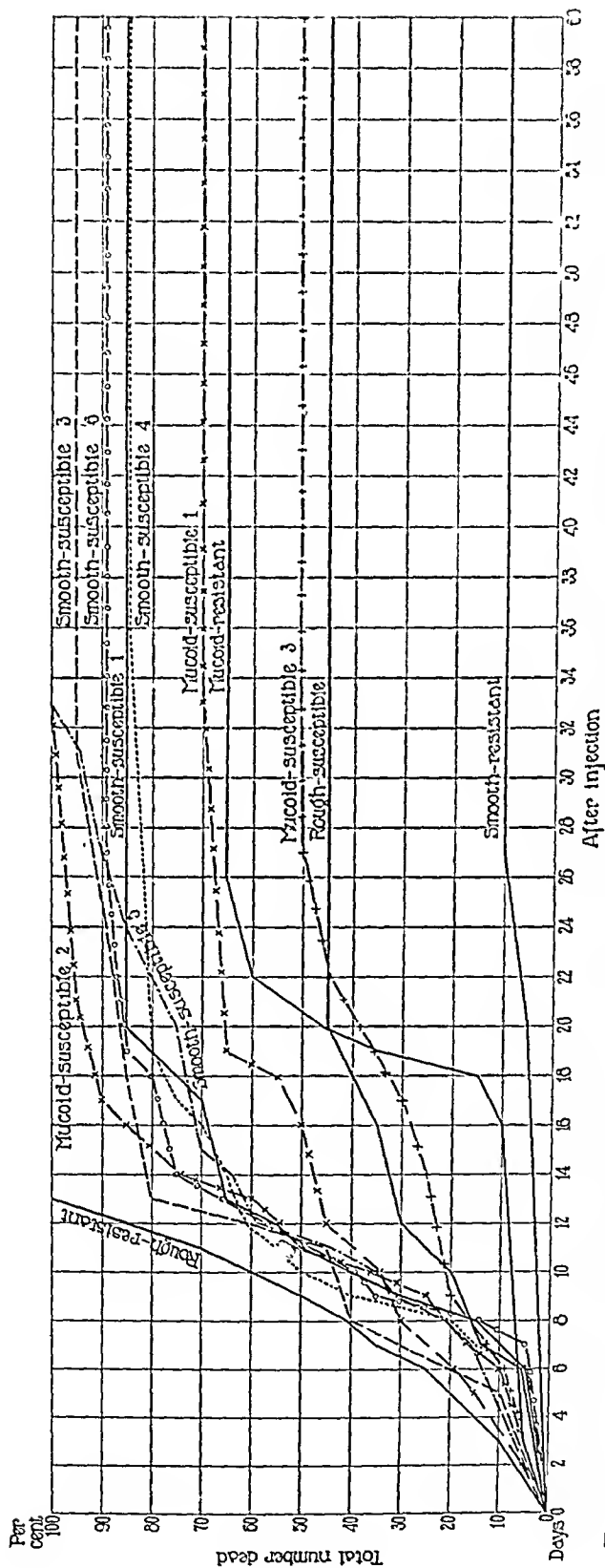
TEXT-FIG. 6. Relative mortality of mice given smooth, mucoid, and rough strains of *B. enteritidis*. Intraperitoneal titrations. Experiment 5.

of the intraperitoneal titrations. A dose of about 138,000,000 of each culture, except the SR 1, killed in 1 day—the mice given this SR 1 strain showed an average survival time of $3\frac{1}{2}$ days. A dose of about 1,382,000 killed the three mice given the SS 1 culture in an average time of 3 days. Those given the RR 1, MR 1, and MS 1 cultures survived on an average of 3, 5, and 6 days, respectively.

The three mice receiving the SR 1 culture in this dilution survived. A dose of about 138,200 bacilli killed the SS 1 group in an average time of 4 days; the RR 1 group in 5 days, and the RS 1 group in 19 days. Those receiving the MS 1, MR 1, and SR 1 cultures survived. The average surviving time of mice receiving 13,820 of the SS 1 bacilli was 8 days; that of a similar dose of the RR 1 bacilli, 7 days, and of the RS 1 bacilli, 15 days. This dosage of MS 1, SR 1, and MR 1 was not fatal. The titration indicates, therefore, that the SS 1 culture, when injected intraperitoneally into mice, is highly virulent, and that its variants, with the exception of the RR 1 strain, are of low pathogenicity.

At autopsy mice receiving the RR cultures showed, instead of RR colonies, the original smooth-susceptible forms in large numbers. Mice from the other groups showed only type-specific colonies.

The results of the *per os* inoculations are shown in Text-fig. 7, and the autopsy protocols in Table IV. The total specific mortality of the various SS cultures varied between 85 and 100 per cent; that of the MS cultures between 50 and 100 per cent; of the RS cultures 45 per cent; the SR cultures 10 per cent; the MR cultures 65 per cent; and the RR cultures 100 per cent. 85 per cent of the mice receiving the SS 1 culture succumbed and showed on agar plate cultures from the various organisms typical smooth colonies of *B. enteritidis*. 90 per cent of the mice receiving the SS 3 culture succumbed. All of these animals showed smooth-susceptible colonies on the cultures taken at autopsy. 85 per cent of the SS 4 group died. From these smooth-susceptible colonies alone were obtained. 100 per cent of the SS 5 group died. Smooth-susceptible colonies were recovered from all. 90 per cent of the SS 6 group succumbed. From these smooth-susceptible colonies were obtained. Thus it is evident that the reverted, smooth-susceptible forms coming from the phage-resistant, smooth, mucoid, and rough variants are similar in virulence to the original smooth-susceptible strain. 90 per cent of the mice receiving the MS 1 culture died. Of these 70 per cent showed *B. enteritidis* cultures at autopsy. Fourteen of the latter showed mucoid-susceptible colonies in pure culture, or in large numbers. From two, smooth-susceptible forms were likewise obtained. All mice receiving the MS 2 culture died and showed on the agar cultures taken from various organs



TEXT-FIG. 7. Relative mortality of mice given smooth, mucoïd, and rough strains of *B. enteritidis*. *Per os* titrations. Experiment 5.

large numbers of mucoid-susceptible colonies. From four animals, however, smooth-susceptible forms were cultured. 75 per cent of the MS 3 series died. Of these 50 per cent showed *B. enteritidis* colonies. In nine mucoid-susceptible forms were obtained, and in two smooth-susceptible forms. The effect of the mucoid-susceptible cultures, therefore, appears less severe than that of the smooth-susceptible forms, and colony type reversion from mucoid- to smooth-susceptible noteworthy. 75 per cent of the mice receiving the RS culture succumbed. From 45 per cent rough-susceptible colonies were obtained in pure

TABLE IV.

Relative Virulence of Smooth, Mucoid, and Rough Strains of B. enteritidis.
Experiment 5.

No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures: colony types
		No.	Per cent	No.	Per cent	
20	SS-1	17	85	17	85	Smooth 17
20	SS-3	18	90	18	90	" 18
20	SS-4	17	85	17	85	" 17
20	SS-5	20	100	20	100	" 20
20	SS-6	18	90	18	90	" 18
20	MS-1	18	90	14	70	Mucoid 14; smooth 2
20	MS-2	20	100	20	100	" 20; " 4
20	MS-3	15	75	10	50	" 9; " 2
20	RS	15	75	9	45	Rough 9
20	SR	7	35	2	10	Smooth-resistant 2
20	MR	17	100	13	65	Mucoid-resistant 12; smooth-resistant 4
20	RR	20	100	20	100	Smooth 20

culture. Seven of the smooth-resistant group died, two of which showed pure cultures of smooth-resistant *B. enteritidis* colonies. 85 per cent of the mucoid-resistant group died, 65 per cent with positive cultures of *B. enteritidis*. From these latter mucoid-resistant colonies were found in twelve, and smooth-resistant in two. All the mice receiving the rough-resistant culture died, and from each animal smooth-susceptible forms were obtained in pure culture. Thus it is seen that the rough-susceptible and mucoid-resistant forms are of comparatively low virulence, and that the smooth-resistant form is per-

haps of lowest pathogenicity. The fact that the rough-resistant culture reverted to smooth-susceptible in the tissues of the mice makes this particular titration of no value, except to show the comparative instability of this colony type.

The results of the several titrations (Experiments 1 to 5) bring out the following facts: (1) The virulence of type-pure, smooth-susceptible cultures was similar and relatively high. Single cell strains fresh from mice, or preserved at 4°C. for more than 2 years, or recovered from so called "reverted variants" after broth or mouse

TABLE V.

Intraperitoneal Injections of Bacteria from Smooth-Susceptible and Smooth-Resistant Colonies.

Experiment 6.

After injection	Group receiving smooth-susceptible organisms						Group receiving smooth-resistant organisms					
	Mouse 1	2	3	4	5	6	1	2	3	4	5	6
hrs.												
1	+++	+++	+++	+++	++++	+++	0	0	0	0	+	0
2	+++	+++	+++	+++	++	++++		0	0	0	0	0
4	++	+++	∞	∞	++++	∞	0	++	0	0	0	0
6	++++	++	+	+++	++	∞	0	0	0	0	0	0
24	0	+++	0	+++	++	∞	0	0	0	0	0	0
48		Dead				Dead						
72			Dead	Dead	Dead							
96	Dead						S	S	S	S	S	S

+ = 1-10 colonies per drop; ++ = 10-20 colonies per drop; +++ = 20-50 colonies per drop; ++++ = 100 colonies per drop; ∞ = 100+ colonies per drop.

S = surviving and well.

passage, behaved essentially alike. Under the conditions of the experiments, little colony type variation of the smooth-susceptible type occurred *in vivo*. (2) The mucoid-susceptible variant proved somewhat less virulent and tended to revert to the original smooth-susceptible form. The rough-susceptible and mucoid-resistant variants behaved in essentially the same manner. (3) The smooth-resistant forms were of very low virulence, and more stable in the tissues of the mouse than the other variants. (4) Finally, the rough-resistant variant, when

type-pure, was likewise of relatively low virulence, but showed a marked tendency *in vivo* to revert to the original smooth-susceptible form.

The titrations described above indicate that the greatest differences in virulence occur between strains from bacteriophage-susceptible and bacteriophage-resistant colonies. An attempt was made to determine the cause of the reduced virulence on the part of the strains from bacteriophage-resistant colonies.

Experiment 6.—Single cell cultures of smooth-susceptible and smooth-resistant strains of No. 1 were taken from the stock slant and grown 18 hours in broth. Each was then diluted 1:1000 in broth and 1 cc. given intraperitoneally to six mice. This dosage determined by the dilution method proved to be 850,000 organisms of the smooth-susceptible culture and 1,250,000 of the smooth-resistant. Immediately after injection, and at 1, 2, 4, 6, and 24 hours thereafter, each mouse's peritoneal cavity was aspirated by means of a short, beveled No. 24 syringe needle, and 1 drop of the exudate spread over the surface of agar plates. The number of colonies resulting and the fate of the two groups of mice are shown in Table V.

1 hour after injection, all mice receiving the smooth-susceptible culture showed more than 100 colonies per drop on the plates. One mouse of the smooth-resistant series showed about ten colonies; the rest were negative. Later the bacteria increased rapidly in the exudate of the first group, while in the smooth-resistant group, cultures remained sterile. All smooth-susceptible mice were dead within 3 days; those receiving the smooth-resistant organisms survived and appeared in good health at the end of 3 weeks.

This test shows that the smooth-susceptible bacteria multiplied in the peritoneal cavity of the host and that the smooth-resistant forms did not. In fact, other tests indicated that within $\frac{1}{2}$ hour after injection the smooth-resistant organisms had practically disappeared from the peritoneal exudate. Hence, one may infer that the same events occur when the bacilli are given by the normal portal of entry, and may interpret the negative stool cultures and negative autopsy cultures of the preceding titrations as being due to the fact that the variant type bacteria did not survive in the intestinal tract or the tissues of the host.

A final experiment was planned to test the possibility of altering the

virulence of smooth-susceptible cells by merely placing them in contact with bacteriophage.

Experiment 7.—The preliminary control tests were carried out as described in the previous paper.² A single cell culture of smooth-susceptible strain No. 1 was grown 18 hours in broth, washed four times in distilled water, and resuspended in 0.2 per cent dibasic sodium phosphate. A uniform suspension was made to contain about 500,000,000 per cc. 27 cc. was placed in contact with 3 cc. concentrated bacteriophage in broth, 27 cc. in another flask with 3 cc.

TABLE VI.

Titration of Bacteriophage Present after Contact with Smooth-Susceptible Cells at 4°C.

Suspension	Dilution of suspension										Control
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
Bacteria plus broth.....	0	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Bacteria plus bacteriophage.....	0	0	0	0	0	0	+	+++	+++	+++	+++

0 = no growth at 6 hours; + = slight growth at 6 hours; ++ = moderate growth at 6 hours; +++ abundant growth at 6 hours.

TABLE VII.

Acid Agglutination of Organisms in Contact with Bacteriophage at 4°C.

Suspension	pH 2.4	3.01	3.18	3.53	4.03	4.25	4.65	5.01	5.31	Control
Organisms plus broth.....	0	0	0	0	0	0	0	0	0	0
Organisms plus bacteriophage.....	0	0	0	++	++	0	0	0	0	0

of broth, and in a third flask, 27 cc. of phosphate was added to 3 cc. of bacteriophage. The three flasks were then kept at 4°C. for 16 hours. Bacterial counts before and after this treatment showed that no multiplication had occurred.

The two suspensions of bacteria plus bacteriophage and broth were then centrifuged and the supernatant filtered and tested, together with the phosphate-phage control for the presence of bacteriophage. The sedimented organisms were

²Webster and Burn, Paper III, p. 880.

TABLE VIII.
Virulence Titration of Smooth-Susceptible Organisms in Contact with Bacteriophage at 4°C.

Mouse group	Results of aspiration 2 hrs. after injection	Results of aspiration 24 hrs. after injection	Duration of life days
10 mice: organisms plus broth; dose 12,000,000	++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++	∞, ∞, ∞, ∞, ∞, ∞, ∞, ∞, ∞, ∞	3, 3, 3, 3, 3, 3, 3, 4, 4, 4
10 mice: organisms plus broth; dose 120,000	++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++	++++, ++++, ++++, ++++, ++++, ++++, ∞, ∞, ∞, ++++, ++++	4, 4, 5, 5, 5, 6, 6, 7, 7, 7
10 mice: organisms plus bacteriophage; dose 10,000,000	++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++, ++++	++++, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	3
10 mice: organisms plus bacteriophage; dose 100,000	++, ++, ++, ++, ++, ++, ++, ++, ++, ++	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	

0 = no organisms per drop; + = 1-10 organisms per drop; ++ = 10-20 organisms per drop; +++ = 20-50 organisms per drop; ∞ = 50+ organisms per drop.

washed four times in distilled water, tested for acid agglutinability in buffers, for lysis in broth, and injected intraperitoneally into mice. Ten mice were given 12,000,000 of the smooth-susceptible bacteriophage organisms, and ten 120,000; ten received 10,000,000 smooth-susceptible control organisms, and ten 100,000. Peritoneal aspirations were made at 2 and 24 hours. The results are shown in Tables VI, VII, and VIII.

The titre of the bacteriophage-phosphate control proved to be 10^{-7} , while that of the supernatant in contact with the smooth-susceptible organisms was 10^{-1} (Table VI). Thus, there was evidence that bacteriophage had been adsorbed by the smooth-susceptible cells at 4°C . (previous paper). These cells agglutinated at pH 3.5 to 4.03 in acid buffers, while the control suspension did not (previous paper) (Table VII). Finally, aspirations made from mice receiving the smooth-susceptible broth control cultures showed that multiplication *in vivo* had occurred, while those from mice receiving the smooth-susceptible bacteriophage cultures showed no multiplication, and after 24 hours no survival of the bacteria. Only one of these latter mice died, while all of the former, 10,000,000 group, died within 4 days, and the 100,000 group in 7 days (Table VIII). Suspensions transferred to broth did not undergo lysis; probably, therefore, they did not lyse *in vivo*.

This experiment indicates, therefore, that bacteriophage, presumably by coating the surface of smooth-susceptible cells, prevents multiplication in the tissues of the animal and therefore renders the organisms practically non-virulent. The *per os* inoculation tests, in which it was found that the bacteriophage-resistant variant colony strains given by the normal portal of entry did not survive within the organism, are probably to be explained in the same way.

DISCUSSION.

The virulence titrations of *B. enteritidis* described in this paper have been rigidly controlled; in most instances, single cell strains were used. They show definitely that cultures from mucoid and rough bacteriophage-resistant colonies are less virulent than those from the typical smooth-susceptible colonies. The results agree with those of Arkwright (3), Topley (4), Bronfenbrenner, Muckenfuss, and Korb (5), Jordan (6), Goyle (7), and others who have studied the paratyphoid-enteritidis group, but are distinctly at variance with d'Hérelle's (8)

statements that cultures from resistant colonies are of greater pathogenicity than those from the typical smooth type colony. Furthermore, the tests indicate that type-pure cultures from the common smooth colonies are of similar virulence, whether obtained from mice in epidemic or endemic periods, or from stock agar slants kept for 2 years at 4°C. In this respect, the results agree in general with those of Smith and Nelson (9) as well as with our own earlier work with mouse paratyphoid (*B. pestis caviæ*) (1, b), and rabbit pasteurella organisms (*Bact. lepi-septicum*) (1, e). Topley and his associates (10), on the other hand, consider that the virulence of smooth type cultures of mouse typhoid bacilli fluctuates to a significant degree. Finally, we have shown that the loss of virulence of variant strains from smooth-, mucoid-, and rough-resistant colonies is due to the fact that they do not multiply in the tissues of the host, and we have suggested the possibility that this inability to grow is due to a coating of the bacilli by bacteriophage.

The data contained in three papers of this series (II, III, IV) show clearly that the colony type transformation from smooth to mucoid and rough is reversible; accordingly they agree with the results of Jordan (6). It must be noted, however, that in this respect the intestinal group of bacteria appears to differ from bacteria of the respiratory tract. For example, *Bact. lepi-septicum*, *B. friedländeri*, *Bact. diphtheriæ*, and streptococci change from smooth to rough, but as yet no demonstration of the reverse transformation from rough to smooth has been made. The nature of the change is not known; in the case of *B. enteritidis* mouse typhoid organisms, it seems to be acquired rather than hereditary; while the transformations of the respiratory tract organisms mentioned above appear relatively permanent. However, the term "mutation" is not justifiable (d'Hérelle), nor does the "life-cycle" theory (Hadley) find support in our own experimental observations. At present, therefore, two general types of transformation may be recognized: the relatively permanent changes associated with organisms of the respiratory tract and the easily reversible changes of intestinal tract bacteria, where temperature, characteristics of medium, and presence of bacteriophage appear to determine the process. A final and complete explanation, however, must await further experimental observations.

The importance of bacterial transformations in determining the amount and severity of disease is, we believe, relatively slight. And this despite the following facts: (1) that variants of low virulence may be derived experimentally under conditions similar to those prevailing in nature and that they revert again to the original virulent forms; (2) that some of these variants are known to exist "naturally;" and (3) that these variants may possibly revert in nature to the forms of high pathogenicity. Far more significant is the fact that these variant strains are neither common nor abundant in nature and that the smooth type colony is generally known to prevail during all phases of disease, endemic and epidemic. Our own studies of experimentally induced epidemics are confirming this belief amply and demonstrating besides that the virulence of these common smooth type colony cultures is at all times relatively constant.

CONCLUSIONS.

1. Single cell mouse strains of *B. enteritidis* from smooth-susceptible colonies were, under the various conditions tested, of the same high degree of virulence. 2 year old cultures, strains obtained both in interepidemic and epidemic periods, and "reverted" strains from variant colonies showed the same degree of pathogenicity.

2. Single cell strains from variant smooth, mucoid, and rough phage-resistant colonies were definitely less virulent than the usual smooth-susceptible strains. The loss of virulence in each instance resulted apparently from contact with bacteriophage which rendered the individual cells incapable of multiplication in the animal tissues.

3. A reduction of virulence was induced experimentally when washed cultures of the highly pathogenic smooth-susceptible cells were exposed to bacteriophage and thus rendered incapable of multiplication in the inoculated animals.

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STUDIES ON BACTERIAL ENZYMES.

VI. THE MALTASE OF THE DIPHTHERIA BACILLUS.

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INTRODUCTION.

It is important to obtain knowledge of the mechanism of maltose fermentation by the diphtheria bacillus since the test for acid production from this sugar is one of the means employed in distinguishing the true etiologic agent of diphtheria from certain other morphologically similar but non-pathogenic bacilli. Previous demonstrations (1-6) of the carbohydrate-hydrolyzing enzymes of pneumococci, meningococci, *botulinus* and Welch bacilli (1-6) have proved that the activity of these enzymes is independent of the presence of the living or formed bacterial cell, and at the same time have furnished indirect evidence that these particular bacteria do not attack higher carbohydrates without preliminary hydrolysis. However, since different species of bacteria may form entirely different products from the fermentation of the same sugar (4), it is desirable to obtain experimental evidence of the carbohydrate-hydrolyzing enzymes of important species of bacteria rather than to assume their presence from the simple observation of acid or gas production by living cultures.

In the present investigation, experiments were made to determine whether or not sterile solutions of the intracellular substances of diphtheria bacilli possess the property of hydrolyzing maltose. The bacterial solutions were filtered through Berkefeld candles after physical disintegration of the bacilli by repeated freezing and thawing, and hence were devoid of cell fragments as well as free from living bacteria.

EXPERIMENTAL.

Methods.

Preparation of Diphtheria Bacillus Enzyme Solutions.—The enzyme solution was prepared from a virulent and recently isolated strain of diphtheria bacilli. The procedure was essentially the same as that employed in the preparation of solutions of other bacteria (meningococci, pneumococci, Welch and *botulinus* bacilli) reported in previous papers of this series (3-6).

6 liters of an 18 hour broth culture were centrifuged, and concentrated suspensions of the bacilli were prepared by resuspending the bacterial sediment from each 250 cc. of culture in a volume of approximately 5 cc. Preliminary experiments proved that diphtheria bacilli were much more resistant to disintegration by freezing and thawing than were any of the different bacteria from which enzyme solutions had been prepared in the previous studies. In view of the difficulty of breaking up the bacilli, an attempt was made to facilitate the process by suspending the bacteria in concentrated salt solution. The bacterial suspension was divided into three portions, and the different lots were frozen and thawed under the following conditions: (1) bacilli suspended in the broth supernatant of the original culture, (2) bacilli suspended in the broth supernatant plus 10 per cent NaCl, (3) bacilli suspended in 10 per cent salt solution without broth. It was necessary to continue the freezing and thawing over a long period but after the process had been repeated between 300 and 400 times, stained films revealed a considerable degree of morphological disintegration and a large amount of amorphous, Gram-negative, bacterial substance. Samples of the suspension of disintegrated bacilli were then centrifuged at high speed and the supernatant tested for dissolved bacterial protein by boiling, in order to avoid the danger of filtering the solution before sufficient bacterial dissolution had occurred. When the tests of the supernatant fluids showed the presence of coagulable protein, the entire lot of the bacterial suspension was centrifuged at high speed for 1 hour to remove most of the undissolved bacterial cells and detritus before filtering; the centrifuged supernatants were then passed through a sterile Berkefeld filter.

The filtered bacterial solutions were perfectly clear and were indistinguishable in appearance from the medium in which the bacilli had originally been suspended (broth and salt solution, respectively). The presence of a considerable amount of dissolved bacterial protein derived from the disintegrated diphtheria bacilli was indicated by the heavy precipitation of coagulated protein when the solutions were boiled. So far as could be detected either by this test or by microscopic examination of the stained films, the addition of 10 per cent salt did not facilitate the disruption of the bacillary bodies. But the boiling test showed that the solutions prepared from the bacteria suspended in broth contained more dissolved protein than did the solution prepared from the bacteria suspended in salt solution alone. Apparently, the presence of broth either facilitates the dissolution of the bacterial cells or renders the liberated bacterial substances more

soluble, for it has been our experience with other bacteria (4-6) that the most active and potent preparations are obtained when broth is used as the suspension medium.

Sterility Controls.—No antiseptics were used; the sterility of each enzyme-substrate mixture was controlled by cultural methods.

Substrates.—20 per cent solutions of the test sugars were boiled for 20 minutes, and then added aseptically to sterile 0.1 M phosphate solution (pH 6.9) in amount sufficient to give a final concentration of 4.0 per cent. The final sugar solutions were distributed into sterile Pyrex tubes for use in the enzyme experiments.

Test of Disaccharide-Hydrolyzing Activity of Sterile Solutions of Diphtheria Bacterial Substances.

Living cultures of the strain from which the sterile bacterial solution was prepared, possessed the usual (7) carbohydrate-fermenting properties of diphtheria bacilli, fermenting the common hexoses and maltose, but not forming detectable amounts of acid from either sucrose or lactose. In the following experiment, tests were made to determine whether or not the sterile solution prepared from the intracellular substances of the diphtheria bacilli possessed the property of hydrolyzing maltose. Tests were included with sucrose as representing a disaccharide not fermented by living diphtheria bacilli and also with lactose as representing a disaccharide which sometimes gives equivocal results in fermentation tests with some strains of the living bacilli (7). Since living cultures of diphtheria bacilli produce acid from glucose and from the other hexoses yielded in the hydrolysis of the disaccharides, tests were also made to determine whether or not the sterile enzyme solution possessed the property of acid formation from hexoses.

Hydrolysis Mixtures.—2 cc. portions of sterile, 4 per cent solutions of maltose, lactose and sucrose were distributed into three series of sterile Pyrex test-tubes, and 1.0 cc. of the sterile diphtheria bacterial solution was added to one tube of each of the substrates. A second series was prepared to serve as controls of the heat lability of the hydrolyzing enzymes by adding the same amount of heat-inactivated (boiled) bacterial extract to each substrate. All of the mixtures were incubated at 37°C. for 72 hours and then tested for the presence of hexoses.

Detection of the Hexose Products of the Enzyme Action.—The sucrose series were tested for reducing sugars with Benedict's solution. The lactose and maltose hydrolysis mixtures (where the substrates themselves are reducing sugars) were tested for hexoses by the biological method described in detail in preceding

papers. The method is based upon the acid fermentation of the hexoses by bacteria which do not ferment the disaccharide from which the hexoses are derived. 1 cc. of each of the test, and of the control hydrolysis mixtures, was put into a series of sterile Pyrex test-tubes; 1 cc. of a suspension of bacteria which produce acid from hexoses but which do not attack the test disaccharide was then added to the different tubes. (An atypical strain of colon bacilli which does not attack maltose was added to the maltose tests; typhoid bacilli which do not attack lactose or sucrose were used for the lactose and sucrose tests.)

TABLE I.

Action of Diphtheria Bacillus Enzyme Solution upon Maltose, Lactose and Sucrose.

Disaccharide hydrolysis by diphtheria bacillus enzymes				Action of diphtheria bacillus enzymes on hexose products
Hydrolysis mixture		pH after action of bacteria used as fermenting agents	Change in pH due to fermentation of hexoses previously formed by diphtheria bacillus enzymes	Acid production
Maltose	Active diphtheria bacterial solution	6.5	1.1	0
	Heat-inactivated bacterial solution	7.6	0.0	0
Sucrose	Active diphtheria bacterial solution	7.6	0.0	0
	Heat-inactivated bacterial solution	7.6	0.0	0
Lactose	Active diphtheria bacterial solution	7.6	0.0	0
	Heat-inactivated bacterial solution	7.6	0.0	0

* Δ pH indicates change (decrease) in pH.

The "fermentation mixtures" (equal portions of hydrolysis test mixture plus the bacterial-fermenting agent) were shaken, incubated in the water bath at 37°C. for 1½ hours and then centrifuged at high speed to remove the bacteria. Colorimetric determinations of the pH of the clear supernatant fluids were made to detect the production of acid. That the changes in reaction of the fermentation mixtures were limited to the acid produced from the hexoses previously formed by the active diphtheria bacillus enzyme was controlled by the tests on the control series of heat-inactivated enzyme plus the test disaccharides.

Experiments were made with the three different lots of diphtheria bacterial solution (*i.e.* solutions prepared from the bacilli suspended in broth alone, from bacilli suspended in broth plus 10 per cent NaCl and from bacilli suspended in 10 per cent NaCl without broth). Only quantitative differences were obtained in the experiments with the different types of bacterial solution, and the results from the tests with the enzyme solution prepared from the bacilli suspended in broth alone are summarized in Table I.

The results of these experiments (Table I) show that the sterile solution of the diphtheria bacterial substances contained an active maltase which is heat-labile. In contrast to the results in the tests of the maltose-enzyme mixtures, no acid was formed by the hexose-fermenting bacteria when added to the previously incubated mixtures of diphtheria bacillus enzymes and lactose or sucrose solution; this fact is evidence that the sterile bacterial solution contained only maltase and no lactase or sucrase. Thus, the carbohydrate-hydrolyzing activity of the solution prepared from the cellular substances of diphtheria bacilli is parallel to the fermenting activity of cultures of the living bacteria, maltose being the only disaccharide hydrolyzed by the bacterial solution just as maltose is the only disaccharide from which living diphtheria bacilli can produce acid. These results indicate that the acid fermentation of higher carbohydrates by diphtheria bacilli depends upon the presence of a hydrolyzing enzyme to split the specific carbohydrate to hexose constituents.

It is also shown in Table I that the diphtheria bacillus enzyme solution which hydrolyzes maltose, is devoid of the property of forming acid from the hexose products of hydrolysis. Living diphtheria bacteria actively ferment glucose and the failure of the sterile bacterial solution to possess the acid-forming property of the living bacilli is in contrast to its retention of the original carbohydrate-hydrolyzing activity of the living bacteria. The retention of the carbohydrate-hydrolyzing activity by sterile solutions of the bacterial substances of diphtheria bacilli and the loss of the hexose-fermenting activity, is the same phenomenon which has been evident in the similar studies of pneumococci, meningococci, Welch and *botulinus* bacilli (3-6). While the activity of the hydrolyzing enzymes is apparently quite independent of the presence of the living or formed bacterial cell, the production of acid from hexoses seems to be more intimately associated with the morphological integrity of the bacteria.

Endocellular Nature of the Maltase of the Diphtheria Bacillus.

The previous demonstration of the diphtheria bacillus maltase was made with solutions of the bacterial substances prepared from the bacilli suspended in salt solution alone, as well as with solutions prepared from bacilli suspended in the supernatant fluid of broth cultures. Since the bacterial extracts prepared from the bacilli suspended in salt solution contained none of the exocellular substances liberated in the culture medium during growth, the presence of an active maltase in this type of bacterial solution is in itself evidence that the maltase is an intracellular derivative of the diphtheria bacillus. In order to show more definitely that the maltase is not also an exocellular product set free during growth of the bacilli, comparative tests were made of the maltose-hydrolyzing activity of filtrates of broth cultures and of solutions of the intracellular substances.

These tests were made by the same procedure as described for the previous experiment. The results showed that while the solution of intracellular substances of the bacilli hydrolyzed maltose, the filtrate of the young broth culture which contained only extracellular products was devoid of maltose-hydrolyzing activity. Thus, the maltase of the diphtheria bacillus is an endocellular constituent of the diphtheria bacillus, agreeing in respect to its intracellular nature with the carbohydrate-hydrolyzing enzymes of meningococci, pneumococci, *botulinus* and Welch bacilli (1-6).

COMMENT.

The preceding experiments have demonstrated that diphtheria bacilli possess a maltose-splitting enzyme which remains operative in sterile, filtered solutions of the intracellular substances which can be liberated by physical disintegration of the bacterial cells. Since it is of endocellular origin, the maltase represents a heat-labile constituent of the diphtheria bacillus cell. The demonstration of the active maltose-hydrolyzing enzyme is of interest from two different points of view.

First, experimental proof of the maltase adds to the knowledge of the mechanism of carbohydrate fermentation by the diphtheria bacillus. The fact that the sterile bacterial solution contains a maltase but

no lactase nor sucrase is in agreement with the fact that the living bacilli ferment maltose but no other disaccharide, and indicates that the production of acid from disaccharides by diphtheria bacilli depends upon the possession of a hydrolase to split the specific disaccharide to its fermentable hexose constituents. Living diphtheria bacilli actively ferment glucose but the sterile bacterial solution although it hydrolyzes maltose is devoid of the property of forming acid from the glucose products. These relations are analogous to those previously found (1-6) with pneumococci, meningococci, Welch and *botulinus* bacilli, and indicate that the fermentation of maltose by the living diphtheria bacillus involves two separate sets of reactions: (1) hydrolysis, (2) fermentation of, or acid production from, the hexose products; only the first reaction being independent of the presence of the living or formed bacterial cell.

Maltase is one of the most labile of the known constituents of bacterial cells and would be inactivated if drastic methods were employed to disintegrate the cells. Hence the fact that maltase can be obtained in an active state in the sterile solutions of the intracellular substances of diphtheria bacilli is also of interest as proof of the possibility of preparing solutions or extracts of the endocellular constituents of these bacteria without loss of their original activity. In the preparation of the sterile solutions of diphtheria bacilli reported in this paper the bacterial cells were disrupted simply by repeated freezing and thawing, which would be expected to yield the intracellular substances in more nearly the original condition in which they exist in the living bacilli than in preparations obtained by dissolving the bacteria in alkali. However, in order to obtain active preparations containing the desired amount of dissolved bacterial substance (protein) it is necessary to repeat the freezing and thawing process 300 to 400 times, since diphtheria bacilli are much more resistant to disintegration by freezing and thawing than are the bacteria studied in previous investigations (1-6). Xerosis bacilli are even more resistant to this treatment than are diphtheria bacilli, and we have been unable to obtain satisfactory solutions of their intracellular substances by freezing and thawing suspensions of these bacteria. The diphtheria bacillus solutions contain sufficient dissolved bacterial protein to be used in immunological studies, and are being employed for that purpose in investigations to be reported in later papers.

SUMMARY.

Diphtheria bacilli possess a heat-labile, endocellular maltase, which retains its activity in sterile solutions of the intracellular substances which are liberated by physical disintegration of the bacterial cells. The proof of the maltase activity and the detection of the hexose products constitute experimental evidence that the living diphtheria bacillus attacks maltose by way of a preliminary hydrolysis.

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STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY.

IV. THE INHERITANCE OF SUSCEPTIBILITY TO TAR-INDUCED TUMORS IN THE LUNGS OF MICE.

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Previous communications from this laboratory have provided evidence in favor of the theory that susceptibility to tumors of the mammary gland (1) and tumors of the lung (2) is inherited. This evidence is based upon observations of spontaneous tumors. The discovery and perfection of methods of inducing tumors of various kinds have demonstrated the important rôle played by chronic irritation in the origin of neoplasms. It is conceivable that the so-called "spontaneous" tumors also are the result of chronic irritation and that they seem to be spontaneous simply because the chronic irritant is unknown to the observer. This is a possible point of view though by no means one necessarily consequent upon the facts at hand. Whatever the relationship between the two kinds of tumors will ultimately prove to be, it has seemed pertinent to investigate the relation between chronic irritation and a demonstrated hereditary susceptibility.

The material used for this purpose consists of two strains of mice chosen from among a number which have been under observation in this laboratory for some years. They are an agouti strain, No. 1194, and the Bagg albinos. A somewhat detailed account of their history and numbers has already been published (2). The Bagg strain has a low rate of mammary gland tumors but a higher rate of lung tumors, while the opposite condition obtains in Strain 1194, which has a higher rate of mammary gland tumors than the preceding group but a low rate of tumors of the lung. The lung tumor rates have been shown (2) to be significantly different in the two stocks. Among 208 mice belonging to Strain 1194 which lived to be at least 12 months old, 6.73 per cent \pm 1.17 had primary neoplasms in the lung. In the Bagg strain 37.04 per cent \pm 2.80, in a total of 135 mice, had

tumors. The difference between the rates is 30.31 ± 3.04 and is undoubtedly significant. A few tumors of other types also have been found, and Strain 1194 produces a small number of epitheliomas of the jaw, but neither strain has developed tumors elsewhere in the skin.

An initial attempt in the investigation of the problem was made by testing the two strains in regard to their reaction to tar painting on the skin (3). The well known method of applying tar extract periodically to the skin between the shoulders was used, and in many cases carcinomas resulted. The percentage of tumors and the rates at which they appeared were practically the same in the two groups. Although the strains differed in respect to their spontaneous tumor rates, they showed no difference in their response to tar painting on the skin. It was pointed out at the time that the spontaneous tumors originated in one type of epithelium, *i.e.*, of the mammary gland (the lung tumor rates had not yet been demonstrated), while the tar painting test was applied to a different tissue—epithelium of the back. It was obvious that it would be a more interesting experiment to attempt to induce tumors in a tissue in respect to which the stock of mice already differed spontaneously. Since then the differences in lung tumor rates have been demonstrated, and the discovery by Murphy and Sturm (4) of a method of inducing tumors in the lung by tar painting presented the possibility of making the desired test.

Experiment 1.—30 agouti mice, 18 males and 12 females, between 2 and 3 months old, were selected from Strain 1194, and an equal number, 19 males and 11 females, from the Bagg albinos. The tar used was similar to that formerly employed, a residue obtained after distillation at 300°C ., from which acids, bases, and phenols had been removed, and which was then dissolved in benzene. The novelty of the method lies in the loci which are subjected to treatment. The applications, instead of being made always on one spot, are distributed to 12 different regions of the body—on the dorsal and ventral surfaces, axilla, groin, neck, etc. The painting is done three times weekly, and four applications are made on each area, so that the treatment lasts approximately 4 months. After an interval of 6 more months, or when the animals are 12 to 13 months old, they are killed.

After such treatment very few mice develop skin tumors. Apparently no single region in the skin is irritated sufficiently to undergo neoplastic changes, but for some reason, as yet undetermined, a certain percentage of individuals will present primary tumors in the

lung. Since mice are subject to "spontaneous" tumors in the lung, it is necessary to differentiate between the spontaneous and tar-

Agouti mice from strain with low incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Jan	Feb	Mar	Apr	May	June	Skin	Lung - gross	Lung - microscopic
1	♀	-	-	-	-	-	+	—	—	—
2	♂	-	-	-	-	-	+	—	—	—
3	♂	-	-	+	-	-	-	—	—	—
4	♂	-	-	-	-	-	+	—	1 nod. in lobe	+
5	♂	-	+	-	-	-	-	—	—	—
6	♀	-	-	-	-	-	+	—	—	—
7	♀	-	+	-	-	-	-	—	—	—
8	♀	-	-	-	-	-	+	—	Min. nod. in lobe	+
9	♀	-	-	+	-	-	-	—	—	—
10	♀	-	-	-	-	-	+	—	Min. nod. in lobe	—
11	♂	-	+	-	-	-	-	—	—	—
12	♂	-	-	-	-	-	+	—	—	—
13	♂	-	-	+	-	-	-	—	—	—
14	♂	+	-	-	-	-	-	—	—	—
15	♂	-	-	-	-	-	+	—	Min. nod. in lobe	+
16	♀	-	-	-	-	-	+	—	—	—
17	♀	+	-	-	-	-	-	—	—	—
18	♂	-	-	-	-	-	+	Epithelioma	—	—
19	♀	-	-	-	-	-	+	—	Min. nod. in lobe	—
20	♂	+	-	-	-	-	-	—	—	—
21	♂	-	-	-	-	-	+	Epithelioma	Min. nod. in lobe	+
22	♂	-	-	-	-	-	+	—	2 . . 2 .	+
23	♂	-	-	+	-	-	-	—	—	+
24	♂	-	-	-	-	-	+	—	—	—
25	♂	-	-	-	-	-	+	—	—	—
26	♂	-	-	-	-	-	+	—	—	—
27	♀	-	-	+	-	-	-	—	—	—
28	♀	+	-	-	-	-	-	—	—	—
29	♀	-	+	-	-	-	-	—	—	—
30	♂	-	-	-	-	-	+	—	Min. nod. in lobe	+

Agouti mice surviving 6 months after tarring { 5 positive
10 negative
1 doubtful

TEXT-FIG. 1.

induced growths. This is done by checking the age of the animals. As a rule, lung tumors do not develop spontaneously until later in

life. In our stocks 8 months is the earliest age at which such growths have been noted, and then in only 1 to 3 per cent of the individuals.

Albino mice from strain with high incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring					Lesions		
		Jan	Feb	Mar	Apr	May	Skin	Lung-gross	Lung-microscopic
31	♂	—	—	—	—	+	—	Many nodules in 4 lobes	+
32	♂	—	—	—	—	+	—	" " " 5 "	+
33	♂	—	—	—	—	+	—	" " " 4 "	+
34	♂	—	—	—	—	+	—	" " " 5 "	+
35	♂	—	—	—	—	+	Hyperplasia	" " " 3 "	+
36	♂	—	—	—	+	—	—	2 " " 2 "	—
37	♂	—	—	—	—	+	—	—	—
38	♂	—	—	—	+	—	—	—	—
39	♂	—	—	—	+	—	Epithelioma	—	—
40	♂	—	—	—	—	+	—	Many nodules in 3 lobes	+
41	♂	—	—	—	—	+	—	" " " 4 "	+
42	♂	—	—	—	—	+	—	" " " 4 "	+
43	♂	—	—	—	—	+	—	" " " 5 "	+
44	♂	—	—	—	—	+	—	2 " " 2 "	+
45	♂	—	—	+	—	—	—	Many " " 3 "	+
46	♂	—	—	+	—	—	—	2 " " 2 "	—
47	♂	—	—	+	—	—	—	Many " " 2 "	—
48	♂	—	—	+	—	—	—	—	—
49	♂	—	—	—	—	+	—	Many nodules in 4 lobes	+
50	♀	—	—	—	—	+	—	" " " 4 "	+
51	♀	—	—	—	—	+	—	2 " " 2 "	+
52	♀	—	—	—	—	+	—	Many " " 5 "	+
53	♀	—	—	—	—	+	—	" " " 5 "	+
54	♀	—	—	—	—	+	—	" " " 4 "	+
55	♀	—	—	—	—	+	—	" " " 4 "	+
56	♀	—	—	—	—	+	—	" " " 5 "	+
57	♀	—	—	—	—	+	—	" " " 4 "	+
58	♀	—	—	—	—	+	—	" " " 4 "	+
59	♀	—	—	—	—	+	—	" " " 3 "	+
60	♀	—	—	—	—	+	—	" " " 4 "	+

Albino mice surviving 6 months after tarring { 22 positive
0 negative
0 doubtful

TEXT-FIG. 2.

The youngest mouse in the Bagg strain in which a lung tumor has been found was 15 months old, and in the agoutis, 18 months. A

marked lowering of the tumor age or increase in tumor rate among mice which have been tarred in the way described may fairly be considered a result of the treatment.

Text-figs. 1 and 2 give the results of the experiment. Almost half of the agoutis died before the expiration of the 6 month interval. In the skin of 2 of the mice neoplastic changes had occurred; both animals proved to have carcinomas. At autopsy minute lesions or nodules were found in the lungs of 8 individuals, usually in only one lobe of the lung. Microscopic examination, however, revealed that only 5 mice had lung tumors, 1 was doubtful, and 10 were negative.

Of the albinos, a larger number lived to the end of the experiment. 1 that died the 4th month after tarring was stopped had an epithelioma on the back. 1 that died in the 3rd month after tarring had a primary lung tumor. All of the 22 mice that were killed after 6 months presented primary tumors of the lung, making a total of 23 positive and none negative. The large majority of them were multiple.

In this experiment the two strains gave different percentages of lung tumors after the tar treatment. Strain 1194, which "spontaneously" produces 6.73 per cent of lung tumors, produced 37.5 per cent when painted with tar. The rate of spontaneous lung tumors in the Bagg strain is 37.04 per cent, but when painted the incidence increased to 100 per cent. In both strains the rate increased, but the rate of the agouti stock did not increase sufficiently to overtake that of the albinos. A difference still exists between them.

The numbers of mice employed were not large, and it was, of course, desirable to repeat the experiment. The results, however, so strongly supported the conception of an hereditary difference in constitution in the animals of the two families that an experiment to test the point was carried out. If an hereditary difference persists even after tarring, then it should be possible to cross the two stocks and with the first filial generation thus obtained make backcrosses to the two original strains and demonstrate differences in the individuals of the backcross generation. In anticipation of the result actually obtained, mice in the first experiment had been crossed before the outcome of the experiment was known, and in the succeeding generation backcrosses were made after the discontinuance of tarring. For each generation a group from each of the original stocks was run at the

same time as controls. The controls are of the same nature as the original experiment and will be summarized after they have been described in detail.

F₁ from high tumor strain (Bagg albino) X low tumor strain (No. 1194)

Number	Sex	Length of life after tarring						Lesions		
		Aug	Sept	Oct	Nov	Dec	Jan	Skin	Lung - gross	Lung - microscopic
201	♂						+		1 lesion in 1 lobe	+
202	•						+		1 " " 1 "	+
203	•						+		2 " " 1 "	+
204	•						+		2 " " 2 "	+
205	•						+		3 " " 3 "	+
206	•	Died during tarring							0 " " 0 "	
207	•						+		3 " " 3 "	+
208	•						+		1 " " 1 "	-
209	•									
210	♀									
211	•									
212	•									
213	•									
214	♂						+		3 " " 3 "	+
215	•						+		3 " " 3 "	+
216	•						+		0 " " 0 "	-
217	♀						+		4 " " 3 "	+
218	•						+		4 " " 3 "	+
219	•						+		3 " " 2 "	+
220	•						+		1 " " 1 "	+
221	♂						+		0 " " 0 "	-
222	•						+		1 " " 1 "	+
223	•						+		3 " " 2 "	+
224	•						+		2 " " 2 "	+
225	•						+		0 " " 0 "	-
226	•						+		2 " " 2 "	+
227	•						+		2 " " 2 "	+
228	•						+		2 " " 2 "	+
229	•						+		5 " " 3 "	+
230	♀						+		5 " " 3 "	+
231	♂						+		2 " " 2 "	+
232	•						+		2 " " 2 "	+
233	♀						+		1 " " 1 "	?
234	•			+					0 " " 0 "	0
235	•						+		3 " " 2 "	+

22 positive - 19%
 4 negative - 14%
 2 doubtful - 7%

TEXT-FIG. 3.

Experiment 2.—Before the conclusion of the tar painting in Experiment 1, crosses between the two strains were made in which all of the females were used and all of the males with the exception of 2 agouti males, Nos. 21 and 24, and 7 albino males, Nos. 43 to 49 inclusive. Many of the crosses, however, were un-

productive of offspring either through the sterility of the parents or through destruction of the young. 13 of the matings were successful. In 6 instances the male parent was from the albino or high tumor strain, and in 7 cases from the agouti or low tumor strain. A few of the next generation were too sickly to be used, but 35 of the F₁ generation were selected to be tested by the same method of tarring as were their parents. The results are shown in Text-fig. 3. The painting was begun when the mice were 2 to 3 months old, as in the previous experiment.

Agouti mice from strain with low incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Aug	Sept	Oct	Nov	Dec	Jan	Glands	Lung - gross	Lung - microscopic
251	♂	+						—	—	—
252	♀						+	—	1 nodule in 4 lobes	—
253	♀						+	Mammary gland tumor	—	—
254	♀				+			—	—	—
255	♀					+		—	1 nodule in 1 lobe	+
256	♂		+					—	No autopsy	—
257	♂						+	—	1 nodule in 1 lobe	—
258	♀						+	—	—	—
259	♂				+			—	—	—
260	♂				+			—	—	—
261	♀						+	—	—	—
262	♀						+	—	—	—
263	♂						+	—	—	—
264	♀						+	—	1 nodule in 1 lobe	+
265	♀						+	—	1 . . 1 .	—
266	♂					+		—	—	—
267	♂						+	—	—	—
268	♀						+	—	—	—
269	♂	+						—	No autopsy	—
270	♀						+	—	—	—

2 positive
11 negative
0 doubtful

TEXT-FIG. 4.

The mortality was very low. 1 male, No. 206, died before the tarring was completed, and 1 female, No. 234, died 3 months after the treatment was over. Neither had a tumor of any kind. 1 male, No. 209, and 4 females, Nos. 210 to 213 inclusive, were not killed with the others. They came from parents both of which had lung tumors and were kept for breeding purposes. In two other matings the parents were positive and the three offspring (Nos. 229, 230, 231) also were positive. In two matings the parents were both negative but they had not lived

the allotted period. Of their offspring four (Nos. 202, 203, 204, 205) were positive and one (No. 206) died during treatment. The rest of the F_1 came from parents one of which had shown a lung tumor in the first experiment and the other had not. There were 28 in all of which 21 were males. At autopsy numerous lesions were discovered in the lung, and upon microscopical examination 22 mice were found to have primary tumors, 4 mice were negative, and 2 were doubtful; that is, 79 per cent had undoubted tumors. All of the negative animals were males.

As controls for this experiment, groups of mice from each of the two original families were submitted to the same experimental treatment. The first group

Albino mice from strain with high incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Aug	Sept	Oct	Nov	Dec	Jan	Skin	Lung - gross	Lung-microscopic
235	♂						†	—	Many nodes in 5 lobes	+
237	♂						†	—	• • 1 •	?
238	♀						†	—	• • 3 •	+
239	♂						†	—	—	—
240	♂				†			—	—	—
241	♂						†	—	Many nodes in 4 lobes	+
242	♂						†	—	• • 1 •	+
243	♂			†				—	—	—
244	♀	†						—	Many nodes in 3 lobes	—
245	♀	Died during tarring						—	—	—
246	♂						†	—	Many nodes in 3 lobes	+
247	♂						†	—	• • 3 •	+
248	♀						†	—	• • 7 •	+
249	♂						†	—	• • 2 •	+
250	♂						†	—	—	—

8 positive
2 negative
1 doubtful

TEXT-FIG. 5.

comprised 20 agouti mice from the low tumor Strain 1194, and the second group consisted of 15 mice taken from the Bagg albino stock. Again the age factor was checked, only mice of 2 to 3 months being used.

Text-figs. 4 and 5 present the results. 13 of the agoutis and 11 albinos lived to practically the end of the experiment. (A few are included which died 2 days to 2 weeks before the end.) A colored female, No. 253, had a mammary gland tumor. There were no lesions in the skin. Of the agoutis, 2 were positive and 11 negative, while among the albinos 8 were positive, 2 negative, and 1 doubtful.

Backcross from F₁ (Begg albino x No 1194) X Begg albino (high tumor strain)

Number	Sex	Color	Length of life after tarring					Lesions		
			Nov	Dec	Jan	Feb	Mar	Apr	Skin	Lung — gross
340	♂	Agouti						+	∞ lesions in 4 lobes	+
341	♂	Cin.	Died during tarring						0	0
342	♂	Albino						+	0	0
343	♂	•						+	2	2
344	♂	•						+	∞	5
345	♂	•						+	4	4
345	♂	•						+	∞	3
347	♀	•						+	4	4
348	♂	•						+	∞	3
349	♂	•						+	3	3
350	♂	•						+	∞	5
351	♂	•						+	0	0
352	♂	•						+	3	2
353	♂	Agouti						+	2	2
354	♂	Albino						+	5	4
355	♂	•	Died during tarring						0	0
356	♀	Agouti						+	3	3
357	♀	Albino						+	6	3
358	♂	•						+	2	2
359	♂	Agouti						+	4	2
360	♂	•	Died during tarring							
361	♂	•						+	4	3
362	♂	Albino						+	0	0
363	♂	•						+	6	3
364	♂	•						+	3	3
365	♂	•						+	0	0
366	♂	•						+	∞	4
367	♂	•						+	0	0
368	♀	Agouti						+	∞	4
369	♂	•						+	∞	5
370	♂	Albino						+	∞	3
371	♂	Black						+	4	3
372	♂	Agouti						+	3	3
373	♂	•						+	2	2
374	♂	Albino						+	5	3
375	♂	Agouti						+	3	2
376	♂	Black						+	6	4
377	♂	Albino						+	∞	4
378	♂	•						+	∞	3
379	♀	Agouti						+	∞	5
380	♂	Albino						+	1	1
381	♂	•						+	2	2

	♂♂		♀♀		
	Pos	Neg	Pos	Neg	Doubtful
Colored	4	0	8	0	1
Albino	9	1	9	3	2
Total	13	1	17	3	3

33 positive - 81%
 4 negative - 11%
 3 doubtful - 8%

TEXT-FIG. 6.

Backcross from F₁ (Bagg albino x No 1194) X No 1194 (low tumor strain)

Number	Sex	Length of life after tarring						Lesions		
		Nov	Dec	Jan	Feb	Mar	Apr	Skin	Lung — gross	lung-microscopic
301	♂						+		3 lesions in 2 lobes	+
302	•						+		0 . . 0 .	—
303	•						+		2 . . 1 .	+
304	♀						+		5 . . 2 .	+
305	•						+		0 . . 0 .	—
306	•						+		5 . . 2 .	+
307	•						+		0 . . 0 .	—
308	•						+		1 . . 1 .	—
309	♂						+		0 . . 0 .	—
310	•						+		∞ . . 3 .	+
311	•						+		2 . . 2 .	—
312	♀						+		2 . . 2 .	—
313	•						+		1 . . 1 .	+
314	•						+		1 . . 1 .	—
315	•						+		3 . . 3 .	—
316	•						+		1 . . 1 .	—
317	•						+		2 . . 2 .	—
318	•						+		0 . . 0 .	—
319	•						+		0 . . 0 .	—
320	•						+		2 . . 2 .	+
321	•						+		2 . . 2 .	—
322	♂						+		0 . . 0 .	—
323	•						+		1 . . 1 .	—
324	•			+					0 . . 0 .	—
325	•						+		1 . . 1 .	—
326	•						+		2 . . 2 .	+
327	•						+		1 . . 1 .	+
328	•						+		1 . . 1 .	+
329	♀						+		1 . . 1 .	+
330	•						+		3 . . 2 .	—
331	•						+		1 . . 1 .	+
332	♂						+		0 . . 0 .	—
333	•						+		2 . . 2 .	+
334	•						+		1 . . 1 .	—
335	•						+		0 . . 0 .	—
336	•						+		∞ . . 2 .	+
337	•						+		0 . . 0 .	—
338	•						+		1 . . 1 .	—
339	•						+		1 . . 1 .	+

♂♂ ♀♀
 Pos. Neg. Pos. Neg.
 9 11 6 12

15 positive = 39%

23 negative = 61%

TEXT-FIG. 7.

In this experiment we may note that the cross between the two stocks gives a high percentage of tumors. Among the controls painted at the same time, the individuals from the high tumor strain showed a greater number of pulmonary tumors than did those from the low tumor strain—just as they had in the first experiment. Among the affected mice, the number of nodules and the number of lobes involved were greater in those from the high tumor stock than in the low.

Agouti mice from strain with low incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring ¹						Lesions		
		Nov	Dec	Jan	Feb	Mar	Apr	Skin	Lung - gross	Lung - microscopic
382	♂						+	—	—	—
383	♀						+	—	1 nod. in 1 lobe	—
384	♀						+	—	2 . . 2 .	+
385	♀						+	—	2 . . 2 .	+
386	♂						+	—	1 . . 1 .	—
387	♂						+	—	1 . . 1 .	—
388	♀						+	—	1 . . 1 .	—
389	♀						+	—	—	—
390	♀						+	—	—	—
391	♂						+	—	2 nod. in 2 lobes	—
392	♂						+	—	—	—
393	♀						+	—	1 nod. in 1 lobe	—
394	♂						+	hyperplasia	1 . . 1 .	—
395	♂						+	—	—	—
396	♂						+	—	1 nod. in 1 lobe	+
397	♀						+	—	—	—
398	♂						+	—	—	—
399	♀						+	—	1 nod. in 1 lobe	—
400	♀						+	—	—	—
401	♀						+	—	—	—

3 positive
 11 negative
 0 doubtful

TEXT-FIG. 8.

Experiment 3.—Four groups of mice at the age of 2 to 3 months were tarred as in the preceding experiment. Two series were mice obtained from backcrosses between the first filial generation and the two original strains, and two series were mice from the original strains and were used as controls.

Group 1. Backcross from the High Tumor Strain.—These mice were obtained by crossing sons belonging to the first filial generation with the Bagg albinos

or high tumor strain. Each male was mated with several females. In a few cases one of the females used was the maternal parent from Experiment 1, but no young suitable for the experiment were obtained from them. The mothers of the offspring used were from stock. They had not been treated by tar painting, so that it is not known how many or which individuals would have been susceptible to tar-induced growths in the lung. 42 offspring were obtained, and the results of tarring are shown in Text-fig. 6. 3 mice died during tarring, and 1 in the 1st and 1 in the 5th month after tarring. At autopsy it was

Albino mice from strain with high incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Nov	Dec	Jan	Feb	Mar	Apr	Skin	Lung-gross	Lung-microscopic
402	♂	—	—	+	—	—	—	—	—	—
403	♂	+	—	—	—	—	—	—	—	—
404	♂	—	—	—	—	—	+	—	1 nodule in 3 lobes	+
405	♀	—	—	—	—	—	+	—	. . . 5 .	+
406	♀	Died during tarring						—	No autopsy	—
407	♂	—	—	—	—	—	+	—	1 nodule in 4 lobes	+
408	♂	—	—	—	—	—	+	—	. . . 2 .	+
409	♂	—	+	—	—	—	—	—	1 . . 1 .	+
410	♂	—	—	—	—	—	+	—	1 nodule . . 3 .	+
411	♂	—	—	—	—	—	+	—	. . . 2 .	+
412	♂	—	—	—	—	—	+	—	1 . . 1 .	—
413	♂	—	—	—	—	—	+	—	1 . . 3 .	+
414	♂	—	—	—	—	+	—	—	—	—
415	♂	—	—	+	—	—	—	—	—	—
416	♂	—	—	—	—	—	+	—	2 nodules in 2 lobes	+
417	♂	—	—	—	—	—	+	—	3 . . 3 .	+
418	♂	—	—	—	—	—	+	—	3 . . 3 .	—
419	♂	—	—	—	—	+	—	—	—	—
420	♂	—	—	—	—	—	+	—	2 nodules in 2 lobes	—

10 positive
4 negative
0 doubtful

TEXT-FIG. 9.

found that the lesions in the affected individuals were very numerous. Among 14 males, all but 1 were positive, while among 23 females, 17 showed tumors of the lung, 3 were without them, and 3 were doubtful. If the two groups are taken together and the 3 doubtful cases classed as negative, the ratio is 30 with tumors and 7 without, or 81.1 per cent tumor mice. This rate is about the same as that of the F_1 and of the albino stock.

Group 2. Backcross from the Low Tumor Strain.—The same F_1 males which were backcrossed to the albino stock were also mated with females from Strain 1194, the low tumor strain. 39 offspring resulted and all were agouti as expected. 1 died the 3rd month after tarring. The ratio (Text-fig. 7) for the males was 9 with tumors to 11 without tumor, for the females, 6 positive to 12 negative. The two classes taken together give 15 positive to 23 negative, or 39.47 per cent. This is only about half the number of tumors found in the F_1 or the backcross group from the high tumor strain.

Group 3. Control from Low Tumor Strain.—20 control mice (9 males and 11 females) were selected from Strain 1194, Text-fig. 8. None died during the treatment. When killed, 1 showed an epithelial proliferation on the back of the neck at one of the painted areas, but it had not progressed far enough to be called an epithelioma. Only 3 of the animals produced tumors in the lung; the other 17 were negative.

Group 4. Control from the High Tumor Strain.—The control from the albino group consisted of 19 mice, of which 17 were males, Text-fig. 9. 6 died before the

	Strain 1194			Bagg albino		
	Tumor	Non-tumor	Doubtful	Tumor	Non-tumor	Doubtful
Exp 1	9	10	1	23	0	0
• 2	2	11	0	8	2	1
• 3	3	17	0	13	4	0
Total	10	28	1	41	6	1

Strain 1194 11 tumor : 28 non-tumor = 22.47% \pm 4.02
 Albino 41 " " = 67.42% \pm 3.43
 Difference = 62.97% \pm 5.29

TEXT-FIG. 10.

end of the experimental period. The lesions in the lung were more numerous than in the preceding group, both as to number of individuals and number of lobes affected. Tumors were found in 9 of the 13 survivors. 1 of the mice dying only 2 months after the discontinuance of the tar treatment had a lung tumor, so that the ratio is 10 to 4. In this case also the albino mice from the high tumor strain give a greater percentage of tar tumors than do the agoutis from the low tumor strain.

In the first experiment samples of mice from two strains which differ in their spontaneous tumor rates were contrasted when subjected to a method of tarring which produces lung tumors. The control groups in Experiments 2 and 3 were repetitions of the same test, so that the three experiments may be compared from that point

of view. In each of the three trials, the colored (low tumor strain) and the albino (high tumor strain) groups differed from each other in their reaction, Text-fig. 10. During the series of experiments the percentage of tumors decreased in the case of the agouti strain but increased in the albinos; however, the percentage was always lower in the agoutis and higher among the albinos. The direction of the difference between the two was constant. Combining the figures for all of the trials gives a ratio of 11 tumors to 38 non-tumors for Strain 1194, or 22.45 per cent ± 4.02 . For the Bagg albinos the ratio is 41 tumors to 7 non-tumors, or 85.42 per cent tumors ± 3.43 . The difference between the per cents is 62.97 ± 5.29 . Since the difference is more than eleven times its probable error, it is undoubtedly significant.

Since a distinct difference in the two families is, therefore, shown, it is interesting to note the results of their crossing. Experiment 2 (Text-fig. 3) shows that the F_1 generation when tarred, as were the parents, gives 79 per cent of tumors. This is almost as high a rate as that given by the more susceptible parent strain—the Bagg albinos, which gave 85 per cent of lung tumors. When the F_1 were backcrossed to the high tumor strain, a high rate (81 per cent) was again shown, but when backcrossed to the low tumor strain, Strain 1194, the rate dropped about half, 39 per cent. It seems evident that the percentage of tumors to be obtained in the lungs of mice after tarring depends upon the strain of mice employed and may be controlled by backcrossing the hybrids to one or the other of the strains used.

The data are insufficient to make possible an accurate determination of the number of hereditary factors involved. From the high tumor rate given by the first filial generation it would seem that at least one factor for susceptibility is dominant or partially so. On the basis of the "dominant hypothesis," however, we might expect a higher percentage than 39 in the backcross. Further generations will settle the question.

In solving the problem of the genes involved, help may possibly be obtained from a more detailed study of the histology of the tumors. The tumors do not all present the same picture. The majority are formed of cuboidal cells, somewhat resembling the epithelium of the terminal bronchi. Others are composed of columnar cells, similar to the cells of bronchial epithelium, which, supported by

strands of connective tissue, thrust out finger-like processes. They are comparable to the papillary cyst-adenomas described by Tyzzer. There are many gradations between the types. Some tumors have not the appearance of being malignant, while others show examples of mitosis and definite infiltration. In one case the cells were elongated almost to a spindle shape and were quite unlike either of the types already mentioned. So far a satisfactory classification of the types which would permit identifying them as different genetic classes has not been worked out. This must remain a problem for future investigation.

Another point to be considered is the adequacy of this method of tarring as a test of susceptibility. A slightly different procedure; such as a longer period of tarring, more frequent applications, a longer period between tarring and autopsy, or even a different tar preparation, might yield a higher percentage of tumors. It is possible that the albino strain which gives positive results in 85 per cent of the cases may really be a 100 per cent strain genetically but not show it because of the ineffectiveness of the method employed. In that case the agoutis also might give a higher per cent than 22. Variations in procedure have not been tested. At present we can merely state the results obtained by the method described.

SUMMARY AND CONCLUSIONS.

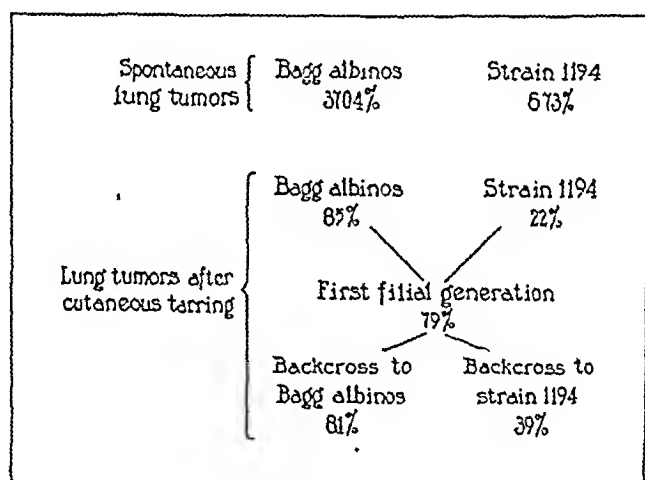
Evidence has previously been submitted in favor of the theory that susceptibility to spontaneous tumors of various types is inherited. The question arose whether susceptibility to tumors induced by tar could be shown to be hereditary by experimenting with the same strains of mice which had already been shown to differ significantly in respect to their spontaneous tumor rates.

Two strains of mice were selected for observation. One strain, the Bagg albinos, has a low rate of mammary gland tumors but a higher rate of spontaneous lung tumors. The other strain, No. 1194, agouti, has a higher rate of mammary gland tumors but a lower rate of tumors of the lung. A previous test showed no difference in the percentages of skin tumors which arose after tar painting.

It has already been shown that the difference in the lung tumor rates

is mathematically significant. When the two stocks are treated with tar by applying the irritant, not in the same spot, but on different areas successively, the percentage of lung tumors is increased in each stock, Text-fig. 11. The rate of the Bagg albinos increased from 37.04 to 85 per cent, that of Strain 1194 from 6.73 to 22 per cent. But a difference between them is still maintained, and this difference also is significant mathematically.

When the two strains are crossed and the offspring subjected to the tar treatment, the latter give a high percentage of lung tumors—79 per cent in 28 individuals—about the same as the parental high tumor strain. When the F_1 sons are backcrossed to the original



TEXT-FIG. 11.

stocks, the cross to the high tumor strain maintains the high tumor rate, 81 per cent in 37 mice, while in the cross to the low tumor strain the percentage drops to 39 per cent in 38 mice. This result indicates that susceptibility to tar-induced tumors in the lung is hereditary. The number of factors concerned has not yet been ascertained. Possibly one or more of them is dominant.

In general, the conception that susceptibility to pulmonary tumors is hereditary seems to be upheld by the fact that the two strains of mice described differ conspicuously in respect to spontaneous tumor rates under ordinary laboratory conditions; the strains differ also

under experimental conditions, as described in this report; and when crossed, their offspring by suitable backcrosses, will again show significant differences.

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FURTHER STUDIES CONCERNING THE FILTRABLE VIRUS PRESENT IN THE SUBMAXILLARY GLANDS OF GUINEA PIGS.

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In a previous paper (1), the occurrence of a filtrable virus in the submaxillary glands of full grown guinea pigs was reported. Emulsions of the submaxillary glands containing potent virus were found to be active after filtration through a Berkefeld candle. The virus was thermolabile, resistant to 50 per cent glycerol, and could not be cultivated either in the presence or absence of air. The various criteria necessary to establish the presence of a filtrable virus were thus fulfilled. It was found extremely difficult, however, to transmit the virus continuously from animal to animal in series. In spite of the fact that young guinea pigs, inoculated intracerebrally,¹ died with great regularity, it was found impossible to transmit the virus from brain to brain. It was only by changing the site of inoculation, as for instance, inoculating first in the brain and then in the testicle, or *vice versa*, that it was possible to carry the virus through two animals in series. In only one instance was the virus transmitted through three young guinea pigs in series alternating as follows: testicle-brain-testicle. In subsequent work the virus has occasionally been transmitted from brain to brain by using very large doses or by making multiple injections. But the second animal in the series in no instance died or was severely sick. The reaction in the second guinea pig of a series, whether the site of inoculation was changed or remained the same, was always very much milder than in the first guinea pig.

In this paper the results of further attempts to transmit the guinea pig virus to other species of animals are reported, certain immuno-

¹ The animals were anesthetized with ether before inoculation.

logical aspects of this disease are discussed, and finally, the description of two methods of transmitting the virus from guinea pig to guinea pig in series is given.

Transmission to Other Species of Animal.

It was thought that if it were possible to infect another species of animal with the guinea pig virus the problem of the transmission of the active agent from animal to animal would become less difficult. It has previously been reported that the intracerebral inoculation of the guinea pig virus into young rabbits, rats, and kittens proved negative. The observations have now been extended to include chickens, pigeons, dogs, a monkey (*Macacus rhesus*), and mice. All these animals, however, proved refractory to intracerebral inoculation¹ of the virus. Direct inoculations of the guinea pig virus into the submaxillary glands of young rabbits and young rats were also made. The glands were removed at various periods following inoculation and examined histologically. No lesions containing nuclear inclusion bodies were found.

Active Immunity.

An important characteristic of filtrable viruses is that in most instances it is possible to produce an active immunity against the agents by the inoculation of small amounts of potent material. The injection of heat-killed virus on the other hand, in contrast with the results obtained with bacteria, fails to produce immunity.

Experimental.—A group of young guinea pigs were divided into two lots; each animal of one lot received three subcutaneous injections of active guinea pig virus on 3 successive days, the guinea pigs of the other lot remained untreated. 2 weeks after the last subcutaneous inoculation, the guinea pigs from both lots were inoculated intracerebrally with active guinea pig virus. The guinea pigs which had received the subcutaneous injections survived, and, with a few exceptions, those which had remained untreated died in the usual period of time. In all the guinea pigs which received the subcutaneous injections of the guinea pig virus and which later proved refractory to brain inoculation, the specific lesion of the virus was found to be present in the submaxillary glands. It was thought, therefore, that the immunity to intracerebral inoculation depended on an active infection of the submaxillary glands and, furthermore, that the virus travelled from the skin to the gland. In order to test this hypothesis, further experiments were undertaken.

It was necessary to determine whether the specific lesion could regularly be found in the submaxillary gland following subcutaneous injections of active guinea pig virus, and if so, how soon after inoculation the lesion was demonstrable. Since infection of the submaxillary gland occurs spontaneously in uninoculated guinea pigs, it was necessary to observe a control group of guinea pigs of approximately the same age over a similar or a longer period of time.

Methods.—The virus used for injection was prepared as follows. The submaxillary glands of two or more adult guinea pigs were removed with sterile precautions. A small piece of each gland was cut off and prepared for histological examination. In experiments in which it was necessary to make injections of virus on 2 or 3 successive days, another piece of each gland was put in 50 per cent glycerol and kept in the ice box until used. The rest of the material was ground thoroughly with sand in a mortar and the emulsion suspended in Locke's solution. Approximately, four glands were suspended in 2 cc. of fluid. The emulsion was centrifuged at low speed for a few moments, and the supernatant fluid used for injection. Aerobic and anaerobic cultures were made from the fluid to make sure that no bacteria were present.

Sections prepared from the submaxillary glands used for injection were always examined to determine whether acidophilic nuclear inclusion bodies were present in the duct cells, and if so, their number and size were noted. Furthermore, 0.1 cc. of the supernatant fluid was inoculated intracerebrally into young guinea pigs to determine the virulence of the particular emulsion. These animals usually died 5 or 6 days following injection and sections were prepared from their brains. In the majority of instances, meningitis was present and the exudate contained cells with acidophilic nuclear inclusion bodies.

Every effort was made to use for inoculation only guinea pigs less than 1 month old. It is, however, extremely difficult to judge the age of guinea pigs by their size. In certain experiments in which it was particularly important to exclude the possibility of a spontaneous infection of the submaxillary glands, only guinea pigs of known age were used. In other experiments where the age of guinea pigs used was uncertain, one submaxillary gland was removed from each guinea pig at the beginning of the experiment² and examined histologically to make certain that the animal was not already infected.

Series E, September 16, 1926.—Seven young guinea pigs, approximately 1 week of age, were selected, and the left submaxillary gland removed from all of them and prepared for histological examination. These guinea pigs were then divided into two groups, three—Nos. 69-7, 69-8, 70-2—were kept together in one cage and left untreated, and four—Nos. 70-3, 70-4, 70-5, 70-7—received three subcutaneous injections on 3 successive days of 0.1 cc. of an emulsion of active guinea pig virus. No definite local reaction occurred at the site of inoculation.

The specific lesion was not found in the left submaxillary glands removed from each guinea pig in both groups at the beginning of the experiment. 6 days after the first subcutaneous inoculation, the right submaxillary gland was removed from Guinea Pig 70-7. Microscopic study of this gland failed to show the specific

² All operations were performed under anesthesia.

lesion. 12 days after the first inoculation, the right submaxillary gland was removed from Guinea Pig 70-4. Microscopic study of this gland showed the presence of typical nuclear inclusion bodies in the duct cells. The inclusion bodies were of the small type, indicating an early lesion. 18 days after the first inoculation the right submaxillary glands were removed from Guinea Pigs 70-3 and 70-5. On histological examination these glands showed typical nuclear inclusion bodies both of the small and large type in the duct cells. 18 days after the beginning of the experiment the right submaxillary glands of the three control guinea pigs were removed. On microscopic study the specific lesion was not found in any of these glands.

This experiment seemed to indicate that young guinea pigs which are injected subcutaneously with active guinea pig virus become infected more rapidly than untreated guinea pigs of approximately the same age and, furthermore, that it requires 12 to 18 days for the specific lesion in the duct cells of the glands to develop. No macroscopic lesion, beyond a little induration, developed at the site of the subcutaneous inoculations.

Further experiments, Series F, K, and R, were made to confirm these observations and to determine whether guinea pigs, which had been injected subcutaneously with virus, subsequently became refractory to intracerebral inoculation. The results of intravenous and intraperitoneal injections of virus were also studied. As controls, a number of guinea pigs were injected subcutaneously with heat-killed virus, and the susceptibility of these animals to intracerebral inoculation was tested. In each experiment, several guinea pigs were not treated, and the submaxillary glands of these animals were removed at the same time that the glands were removed from the inoculated animals.

Table I shows the results of subcutaneous, intraperitoneal, and intravenous injection of guinea pig virus. It will be seen that one guinea pig in the control group, No. 73-2, was already infected at the beginning of the experiment, but that none of the other guinea pigs in this group developed the specific lesion during the time they were under observation.

In none of the guinea pigs of the other groups were lesions present at the beginning of the experiment. 13 to 14 days after inoculation, however, acidophilic inclusion bodies were present in the right submaxillary glands of all these guinea pigs with the exception of one, No. 74-2. In this animal, however, it was doubtful whether the whole dose was injected into the vein.

Many of the guinea pigs in this experiment were greatly weakened by the two operations for the removal of the submaxillary glands, and died of intercurrent infections. Three guinea pigs, Nos. 73-1, 73-3, and 73-5, in the control group, and three guinea pigs, Nos. 71-9, 72-1, and 72-7, which survived the two operations, were inoculated intracerebrally with 0.1 cc. of active guinea pig virus. Guinea Pigs 73-1, 73-3, and 73-5, developed meningitic symptoms and were moribund on the 8th day. Sections prepared from the brains of these animals showed a typical mononuclear, meningeal exudate with cells containing acidophilic nuclear inclusion bodies. Guinea Pigs 71-9, 72-1, and 72-7 remained well.

TABLE I.
Series F.

G. P.	Removal of gland at beginning of experiment					Removal of other gland					
	Gland	Date	Section	Result	Inoculum: virus	Site	Gland	Date	Day	Section	Result
73-1	Left	Oct. 2	646b	Neg.	0, controls		Right	Oct. 15	13th	694b	Neg.
73-2	"	"	647b	Pos.			"	"	"	695b	Pos.
73-3	"	"	648b	Neg.			"	"	"	696b	Neg.
73-4	"	"	649b	"			"	"	"	697b	"
73-5	"	"	650b	"			"	"	"	698b	"
73-6	"	"	651b	"			"	"	"	699b	"
73-8	"	"	653b	"			"	"	"	700b	"
71-0	"	"	655b	"			"	"	"	701b	"
71-8	"	Oct. 1	633b	"	Oct. 1, 2 0.1 cc.	Subc.	"	"	14th	687b	Pos.
71-9	"	"	639b	"	"	"	"	"	"	688b	Pos.
72-1	"	"	636b	"	"	"	"	"	"	689b	Pos.
72-7	"	"	642b	"	"	"	"	"	"	692b	Pos.
72-2	"	"	637b	"	Oct. 1	I.P.	"	"	"	690b	Pos.
72-6	"	"	641b	"	"	"	"	"	"	691b	Pos.
72-8	"	"	643b	"	"	"	"	"	"	693b	Pos.
71-1	"	Oct. 2	656b	"	"	I.V.	"	"	13th	702b	Pos.
71-2	"	"	657b	"	"	"?	"	"	"	703b	Neg.
71-3	"	"	658b	"	"	"	"	"	"	704b	Pos.
71-4	"	"	659b	"	"	"	"	"	"	705b	Pos.

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of submaxillary glands.
 Negative indicates that no lesions were found in the glands.

TABLE II.
Series K.

G. P.	Removal of gland at beginning of experiment					Removal of the other gland					
	Gland	Date	Section	Result	Inoculum: virus	Gland	Date	Day	Section	Result	
90-4	Left	Nov. 18	803b	Neg.	0, controls	Right	Dec. 3	15th	894b	Neg.	
90-5	"	"	804b	"		"	"	"	895b	"	
90-6	"	"	805b	"		"	"	"	896b	"	
90-7	"	"	806b	"		"	"	"	897b	"	
90-8	"	"	807b	"		"	"	"	898b	"	
90-9	"	"	808b	"		"	"	"	899b	"	
91-0	"	"	809b	"		"	"	"	900b	"	
91-1	"	"	810b	"		"	"	"	901b	"	
91-2	"	"	811b	"		"	"	Nov. 29	11th	862b	"
91-3	"	"	812b	"		"	"	Dec. 3	15th	902b	"
91-4	"	"	813b	"		"	"	"	"	903b	"
91-5	"	"	814b	Pos.		"	"	"	"	904b	Pos.
91-6	"	"	815b	Neg.	0.1 cc.	"	"	"	905b	Pos.	
91-7	"	"	816b	"		"	"	"	906b	Pos.	
91-8	"	"	817b	"		"	"	"	907b	Pos.	
91-9	"	"	818b	"		"	Nov. 27	9th	859b	Pos.	
92-0	"	"	819b	"		"	Dec. 2	14th	883b	Pos.	
92-1	"	"	820b	"		"	Dec. 3	15th	908b	Pos.	
92-2	"	"	821b	"		"	"	"	909b	Pos.	
92-3	"	"	822b	"		"	"	"	910b	Pos.	
92-4	"	"	823b	"		"	Nov. 27	9th	860b	Neg.	
92-5	"	"	824b	"		"	"	"	861b	Pos.	
92-6	"	"	825b	"		"	Dec. 3	15th	911b	Pos.	
92-7	"	"	826b	"		"	"	"	912b	Pos.	

					Heat-killed											
					Nov. 19, 20	0.1 cc.	Subc.									
93-1	"	Nov. 19	830b	"	"	"	"	"	"	"	"	"	"	"	913b	Neg.
93-3	"	"	832b	"	"	"	"	"	"	"	"	"	"	"	914b	"
93-4	"	"	833b	"	"	"	"	"	"	"	"	"	"	"	915b	"
93-5	"	"	834b	"	"	"	"	"	"	"	"	"	"	"	916b	"
93-6	"	"	835b	"	"	"	"	"	"	"	"	"	"	"	917b	"
93-7	"	"	836b	"	"	"	"	"	"	"	"	"	"	"	918b	"
93-8	"	"	837b	"	"	"	"	"	"	"	"	"	"	"	919b	"

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.

Negative indicates that no lesions were found in the glands.

Table II shows the results of subcutaneous injections of active and heat-killed guinea pig virus. It has previously been shown (1) that the guinea pig virus is inactivated by exposure to a temperature of 56°C. for a period of 1 hour.

It will be seen from Table II that whereas all except one of the animals injected with active virus developed lesions in the submaxillary glands, none of the guinea pigs injected with heat-killed virus showed acidophilic nuclear inclusion bodies in the duct cells of the glands.

On repetition of this experiment (Series R), similar results were obtained. None of the guinea pigs injected with heat-killed virus developed lesions of the submaxillary glands, in contrast to those animals which had been injected with active virus. The guinea pigs treated with heat-killed material succumbed to intracerebral inoculation of active virus, whereas the animals which had been injected subcutaneously with potent material, survived the same intracerebral injection. Sections prepared from the brains of the animals that died showed a typical meningeal exudate containing cells with acidophilic nuclear inclusion bodies.

Summary of Experiments. Series F, K, R.—The submaxillary glands of twenty-seven young guinea pigs which served as controls in these experiments were examined for the specific lesion. Three of these guinea pigs were found to be infected with the virus at the beginning of the experiment. None of the other twenty-four guinea pigs developed the lesion during the 2 weeks that they were under observation. Although it is impossible to be certain that a submaxillary gland is absolutely negative without cutting serial sections, further evidence that these guinea pigs were actually not infected is brought by the fact that they remained susceptible to intracerebral inoculation of the guinea pig virus. With the exception of the three guinea pigs mentioned above, which were spontaneously infected, all the guinea pigs in this group became sick following the injection of guinea pig virus into the brain. Histological sections of the brains of twelve of the twenty-four guinea pigs showed a typical meningeal exudate.

The submaxillary glands of twenty-two guinea pigs which received either one or two subcutaneous inoculations of active virus, were examined for the presence of acidophilic nuclear inclusions in the duct cells at intervals varying from 9 to 15 days following injection. With the exception of three cases, in one of which the animal died early (9th day), the submaxillary glands of these guinea pigs showed the specific lesion. The immunity of seventeen of these guinea pigs

was tested by intracerebral inoculation, twelve survived, three died acutely 1 or 2 days after inoculation, and two died late from intercurrent infections.

The submaxillary glands of three guinea pigs which had received one intraperitoneal inoculation of active guinea pig virus, were examined 14 days following injection, and all showed acidophilic nuclear inclusion bodies in the duct cells.

The submaxillary gland of four guinea pigs which had received one inoculation of active guinea pig virus intravenously, were examined 13 days following injection. Acidophilic nuclear inclusion bodies were found in three of them. The submaxillary gland in one case was found to be negative, but it is questionable whether, in this instance, the whole dose was injected into the vein.

The submaxillary glands of fourteen guinea pigs which received one or two subcutaneous inoculations of heat-killed virus were examined 14 days after inoculation. No acidophilic nuclear inclusion bodies were found in any of them. The immunity to intracerebral inoculation was tested in seven of these guinea pigs. They all developed meningitic symptoms. Sections prepared from the brains of three of these animals showed a typical meningeal exudate with acidophilic nuclear inclusion bodies.

It was thought of interest to determine whether young guinea pigs from which both submaxillary glands were removed and which were then injected subcutaneously with active guinea pig virus, would also become refractory to intracerebral inoculation.

Series L. December 1, 1926.—Fourteen young guinea pigs, all of approximately the same age, were selected and divided into two groups. Both submaxillary glands were removed from every guinea pig in Group I. The right submaxillary gland was removed from every guinea pig in Group II. On subsequent histological examination, it was found that none of the submaxillary glands removed at the beginning of the experiment showed the specific lesion. All the guinea pigs in Group I and in Group II received two subcutaneous injections on 2 successive days, December 2 and 3, of 0.1 cc. of active guinea pig virus.

12 to 15 days after the first subcutaneous injection, the left submaxillary gland was removed from every guinea pig in Group II. Microscopic study of these glands showed that in every instance the specific lesion had developed following the subcutaneous inoculation of the virus.

On December 17, 15 days after the first subcutaneous inoculation, all the

guinea pigs in Groups I and II which survived were inoculated intracerebrally with 0.1 cc. of active guinea pig virus. Furthermore, two guinea pigs, Nos. 85-6 and 85-8, which had had both submaxillary glands removed 6 weeks before, but which had received no injections, as well as one young normal guinea pig, No. 10-38, were also inoculated into the brain. These three animals, Nos. 85-6, 85-8, and 10-38, all showed typical symptoms and were moribund on the 6th day. Sections prepared from the brains of these animals showed typical lesions with cells containing acidophilic nuclear inclusion bodies. None of these guinea pigs in Groups I and II became sick.

The previous experiments had seemed to indicate that intracerebral immunity depended upon the localization of the virus in the submaxillary gland, but the result of this experiment showed that the presence of the glands was not essential for the development of immunity. An attempt was therefore made to determine whether in this case another localization of the virus had occurred.

In addition to the submaxillary glands, which lie fairly deep in guinea pigs, there is a superficial salivary gland which lies directly over the jugular vein and which probably corresponds to the parotid³ in man. In the majority of full grown guinea pigs in which the submaxillary glands show the specific lesion no acidophilic nuclear inclusion bodies are present in this superficial gland and emulsions of this gland are not infectious when inoculated into the brains of young guinea pigs. The parotid glands of four guinea pigs of Group I were examined, and the specific lesion was found in three, and it seems, therefore, that, in the absence of the submaxillary glands, the virus localizes in the parotid glands, and the animals then become refractory to intracerebral injection. Theoretically, it would be of interest to inject guinea pigs from which both the parotid and submaxillary glands had been removed, to see where the virus would localize under these circumstances, but the position of the parotid glands is such that it is impossible to remove them completely without ligating both jugular veins.

On repetition of this experiment (Series Q) similar results were obtained. Seven guinea pigs from which both submaxillary glands had been removed, but which had received no injections of guinea pig

³ To make the description more simple this gland has been called "parotid gland" in the discussion which follows.

virus, remained susceptible to brain inoculation. Four guinea pigs which had had both submaxillary glands removed and had received one injection of active guinea pig virus were refractory to intracerebral injection, and the specific lesion could be demonstrated in the parotid glands in three of these four animals.

The parotid glands of two guinea pigs which had one submaxillary gland left when they were injected subcutaneously with active guinea pig virus, showed acidophilic nuclear inclusion bodies, both in the submaxillary and the parotid glands. It seems, therefore, that following injections of active virus the parotid glands became infected even though one submaxillary gland was still present. When the infection occurs spontaneously, however, the submaxillary glands and not the parotid glands are usually the first to be infected. This point is brought out by the following experiment.

Series O. December 28, 1926.—The right submaxillary glands were removed from four young guinea pigs, Nos. 10-61, 10-63, 10-64, 10-65. These four animals were kept together in the same cage and left untreated. The left submaxillary glands were removed at various intervals and examined histologically. No acidophilic nuclear inclusion bodies were found in any of the sections prepared from the submaxillary glands removed at the beginning of the experiment. On February 1, 1927, approximately 1 month after the beginning of the experiment, the left submaxillary and parotid glands were removed from Guinea Pig 10-63. The specific lesion was found in the sections prepared from the submaxillary glands, but not in those prepared from the parotid gland.

On March 1, 1927, approximately 2 months after the beginning of the experiment, the left submaxillary and parotid glands were removed from Guinea Pig 10-64. The specific lesion was found in sections prepared from the submaxillary gland, but not in those from the parotid gland.

On March 25, 1927, almost 3 months after the beginning of the experiment, the left submaxillary and parotid glands were removed from Guinea Pigs 10-61 and 10-65. The specific lesion was found in the sections prepared from the submaxillary gland of Guinea Pig 10-65. In those prepared from the parotid gland of this guinea pig an area of reaction was present, but no acidophilic nuclear inclusion bodies were found. The specific lesion was found neither in the submaxillary nor in the parotid gland of Guinea Pig 10-61.

On March 25, 1927, after the removal of the left submaxillary gland and the left parotid gland, Guinea Pigs 10-61 and 10-65 received an intracerebral inoculation of active guinea pig virus. Guinea Pig 10-61 succumbed on the 6th day and the histological examination of the brain showed a typical meningitic exudate with cells containing acidophilic nuclear inclusion bodies. Guinea Pig 10-65 remained well.

The problem of spontaneous infection in this disease is an extremely difficult one. In the experiment reported above, three of four guinea pigs became infected in periods varying from 1 to 3 months. The other guinea pig, though closely confined in the same cage with these animals, never developed the specific lesion in either the submaxillary or parotid glands, and remained susceptible to intracerebral inoculation of the guinea pig virus when more than 3 months of age. In certain other experiments it was thought possible to exclude the occurrence of spontaneous infections of the submaxillary gland, by using only guinea pigs of known age. In one instance, however, the submaxillary gland of a guinea pig 22 days old was found to be infected. There are apparently other factors besides those of age and exposure to the virus that determine whether or not the submaxillary glands of guinea pigs become infected with this disease.

Passive Immunity.—It has been impossible to demonstrate any virucidal properties in the sera of full grown guinea pigs which have been spontaneously infected, or in young guinea pigs which have developed the infection in the submaxillary glands as the result of subcutaneous injections. The failure to show virucidal action in the serum of immune animals in this disease can probably be attributed to the fact that the quantitative factors have not been adequately controlled in these experiments. Further work needs to be done on this phase of immunity.

In the first two experiments on this subject, equal quantities of guinea pig virus and the pooled serum from three full grown guinea pigs, which were subsequently shown to be carrying the infection in their submaxillary glands, were combined and allowed to stand at room temperature for 30 minutes and 0.2 cc. of the mixture was then injected intracerebrally into young guinea pigs. In the same way the serum of a very young guinea pig, 6 days old, whose submaxillary gland did not show the specific lesion, was combined with an equal quantity of guinea pig virus, and injected intracerebrally into young guinea pigs. As additional controls, young guinea pigs were also injected intracerebrally with 0.2 cc. of a mixture of equal quantities of virus and Locke's solution.

In these experiments, even when the dose of virus used was small, the sera of the full grown guinea pigs did not prevent death or the

formation of a meningeal exudate containing cells showing acidophilic nuclear inclusion bodies.

Transmission of the Guinea Pig Virus in Series; from Skin to Submaxillary Gland and from Submaxillary Gland to Skin.—The regularity with which the specific lesion appeared in the submaxillary glands following subcutaneous inoculation of the virus was so striking that it was thought that this might be an easy way to transmit the virus from guinea pig to guinea pig in series. In this experiment, only pigs of known age, which had been born on the premises, were used. In order to control the experiment as accurately as possible, each litter of guinea pigs was, whenever possible, divided into two lots. Two guinea pigs born in one litter were inoculated subcutaneously with an emulsion of virulent submaxillary glands from full grown guinea pigs. As a control two other pigs of the same litter were inoculated with an emulsion of the submaxillary glands obtained from a full grown rabbit. 2 weeks after the subcutaneous inoculation, the submaxillary glands were removed from the guinea pigs injected with guinea pig virus, a piece was taken from each gland for section, and the remainder of the glands emulsified and injected subcutaneously into other guinea pigs of known age. In the same way the submaxillary glands were removed from the two pigs which had been injected subcutaneously with rabbit submaxillary gland, a piece was taken for histological section, and the rest was emulsified and injected subcutaneously into guinea pigs of known age. After 2 weeks the same steps were repeated. In this way the virus was carried through seven generations, the submaxillary glands of the previous generation always being injected subcutaneously into the guinea pigs of the next generation. The control series has been continued exactly in the same way as the series started with the guinea pig virus. In order to determine the virulence of the material used for subcutaneous inoculation in each generation, intracerebral inoculations were also made. Young pigs inoculated intracerebrally with the submaxillary glands of the series started with guinea pig virus have become sick and, in many instances, died. Histological examination of the brains of these animals have shown a typical meningitic exudate containing cells with nuclear inclusion bodies. Guinea pigs inoculated intracerebrally with an emulsion of the submaxillary glands removed from

TABLE III.

I generation							
Subc. inoc.	Inoculum	Date	G. P.	Litter	Age days	Submax. gland	
						Section	Result
0.1 cc.	Submax. glands from 2 old guinea pigs	Nov. 8	87-1	A	4	768b	Pos.
		Nov. 9	87-2	"	"		
				87-5	B		
Removal of glands		Nov. 22	87-1	A	18	838b	Pos.
			87-2	"	"	839b	Neg.
			87-5	B	"	840b	Pos.
II generation							
Subc. inoc. 0.1 cc.	87-1 87-2 87-5	Nov. 22	94-7	D	7		
		Nov. 23	94-8	"	"		
			94-9	E	"		
Removal of glands		Dec. 7	94-7	D	22	927b	Pos.
			94-8	"	"	928b	Pos.
			94-9	E	"	929b	Pos.
III generation							
Subc. inoc. 0.1 cc.	94-7 94-8 94-9	Dec. 7	47	G	2		
		Dec. 8	48	"	"		
			49	F	8		
Removal of glands		Dec. 21	47	G	16	983b	Pos.
			49	F	22		
			48*	G	16		
IV generation							
Subc. inoc. 0.2 cc.	47 49	Dec. 21	10-42	H	7		
				10-43	"		
Removal of glands		Jan. 5	10-42	H	22	1027b	Pos.
			10-43	"	"	1028b	Pos.

* No. 48 used for section only.

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.

Negative indicates that no lesions were found in the glands.

TABLE III—*Concluded.*

V generation							
Subc. inoc.	Inoculum	Date	G. P.	Litter	Age	Submax. gland	
						Section	Result
Subc. inoc. 0.2 cc.	} 10-42 10-43	Jan. 5	} 10-75 10-76 10-77	J	4	1068b	Pos.
				"	"		
				I	13		
Removal of glands		Jan. 20	} 10-75 10-76 10-77	J	19		
				"	"		
				I	28		
VI generation							
Subc. inoc. 0.2 cc.	} 10-75 10-76 10-77	Jan. 20	} 11-06 11-07	K	5		
				"	"		
Removal of glands		Feb. 3	} 11-06 11-07	"	19	1107b	Pos.
				"	"	1108b	Pos.
VII generation							
Subc. inoc. 0.2 cc.	} 11-06 11-07	Feb. 3	} 11-36 11-37	H	6		
					L	8	
Removal of glands		Feb. 17	} 11-36 11-37	H	20	1148b	Pos.
				L	22	1149b	Pos.

the animals of the control series have usually remained well. In certain instances, when these guinea pigs died of other causes, the histological examination of the brain proved negative. The sections of the submaxillary glands of the guinea pigs of the series started with guinea pig virus have in every instance, except one, shown the specific lesion. On the other hand sections from the glands of the first six animals of the control series were all negative. The seventh animal of the series, however, Guinea Pig 11-41, though only 22 days old, developed a spontaneous infection. The guinea pigs in the control series were kept together in a separate cage, but no special precautions, such as sterilizing the cage, were taken. The guinea pigs were allowed

TABLE IV.
Control Series Started with Rabbit Submaxillary.

I generation								
	Inoculum	Date	G. P.	Litter	Age	Submax. gland		
						Section	Result	
Subc. inoc. 0.1 cc.	Rabbit	Nov. 2	87-3	A	4	769b	Neg.	
			87-4	"	"			
		Nov. 9	87-6	B	"			
			87-7	"	"			
Removal of glands		Nov. 23	87-3	A	19	841b	"	
			87-4	"	"	842b	"	
			87-6	B	"	843b	"	
			87-7	"	"	844b	"	
II generation								
Subc. inoc. 0.1 cc.	87-3 87-6 87-7	Nov. 23	95-2	E	8			
		Nov. 24	95-3	D	"			
			95-4	E	"			
Removal of glands		Dec. 7	95-2	E	22	930b	Neg.	
			95-3	D	"	931b	"	
			95-4	E	"	932b	"	
III generation								
Subc. inoc. 0.1 cc.	95-2 95-3 95-4	Dec. 7	52	G	2			
		Dec. 8	53	F	8			
			54	"	"			
Removal of glands		Dec. 21	53	"	22	984b	Neg.	
			54	"	"			
IV generation								
Subc. inoc. 0.2 cc.	53 54	Dec. 21	10-44	H	7			
				10-45	"	"		
Removal of glands		Jan. 5	10-44	"	22	1029b	Neg.	
V generation								
Subc. inoc. 0.2 cc.	10-44	Jan. 5	10-81	I	13			
				10-82	"	"		
				10-80	J	4		
Removal of glands		Jan. 20	10-81	I	28	1069b	Neg.	
			10-82	"	"			
			10-80	J	19			

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.

Negative indicates that no lesions were found in the glands.

TABLE IV—*Concluded.*

VI generation							
	Inoculum	Date	G. P.	Litter	Age <i>days</i>	Submax. gland	
						Section	Result
Subc. inoc. 0.2 cc.	} 10-81 10-82 10-80	Jan. 20	11-08	K	5		
Removal of glands		Feb. 3	11-08	"	19	1109b	Neg.
VII generation							
Subc. inoc. 0.2 cc.	11-08	Feb. 3	} 11-40 11-41	M	6		
				L	8		
Removal of glands		Feb. 17	} 11-40 11-41	M	20	1150b	Neg.
				L	22	1151b	Pos.

to remain with their mothers for 1 week after birth. It is possible that, if these factors had been more carefully controlled, the spontaneous infection of Guinea Pig 11-41 might not have occurred. The results of these experiments are shown in Tables III and IV.

Transmission of the Guinea Pig Virus from Submaxillary Gland to Submaxillary Gland.—The guinea pig virus has also been transmitted through seven generations in series by direct inoculation from gland to gland.

In this experiment guinea pigs of known age were not available. In every instance one submaxillary gland was removed at the beginning of the experiment and prepared for histological examination. In the first generation, Guinea Pig 10-10 was given a large injection of guinea pig virus subcutaneously. The left submaxillary gland was removed 12 days after inoculation, a small piece was prepared for histological section, and the rest emulsified and used for injection. In all subsequent generations injections were made directly into the left submaxillary gland. 14 to 17 days following inoculation, the injected submaxillary gland was removed, a piece prepared for histological section, and the rest placed in 50 per cent glycerol until cultures could be made to exclude the possibility of

TABLE V.

I generation												
Removal of gland at beginning of experiment							Removal of other gland					
G. P.	Gland	Date	Section	Result	Inoculum	Site	Gland	Date	Day	Section	Result	Time in glycerol
10-10	Right	Dec. 8	945b		0.7 cc. virus	Subc.	Left	Dec. 20	12	980b	Pos.	24 hrs.
II generation												
10-48	Right	Dec. 21	985b	Neg.	Left submax. G. P. 10-10	Left submax.	Left	Jan. 4	14	1024b	Pos.	24 hrs.
10-49	"	"	986b	"		"	"	"	"	1025b	Pos.	"
10-50	"	"	987b	"		"	"	"	"	1026b	Neg.	"
III generation												
10-66	Right	Jan. 5	1030b	Neg.	Left submax. G. P. 10-48, 10-49, 10-50	Left submax.	Left	Jan. 22	17	1071b	Neg.	6 days
10-68	"	"	1032b	"		"	"	"	"	1072b	Pos.	"
10-69	"	"	1033b	"		"	"	Jan. 17	12	1056b	Pos.	"
10-70	"	"	1034b	"		"	"	Jan. 22	17	1073b	Pos.	"
10-71	"	"	1035b	"		"	"	"	"	1074b	Pos.	"
IV generation												
11-24	Right	Jan. 28	1093b	Neg.	Left submax. G. P. 10-66, 10-68, 10-70, 10-71	Left submax.	Left	Feb. 14	17	1139b	S. pos.	0
11-25	"	"	1094b	"		"	"	"	"	1140b	S. pos.	0

V generation

4a	Right	Feb. 14	1141b	Neg.	Left submax. G.P. 11-24, 11-25	Left submax.	Left	Mar. 1	15	5c	S. pos. P. neg.	24 hrs.
5a	"	"	1142b	"		" "	"	"	"	6c	S. pos. P. pos.	"

VI generation

49a	Right	Mar. 2	8c	Neg.	Left submax. G.P. 4a, 5a	Left submax.	Left	Mar. 16	14	45c	S. pos. P. neg.	24 hrs.
51a	"	"	10c	"		" "	"	"	"	46c	S. " P. "	"

VII generation

32-3	Right	Mar. 17	47c	Neg.	Left submax. G.P. 49a, 51a	Left submax.	Left	Apr. 2	15	79c	S. pos. P. neg.	
32-4	"	"	48c	"		" "	"	Mar. 24	7	67c	S. pos.	

Left submax. = left submaxillary gland; S. = submaxillary gland; P. = parotid gland.

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.

Negative indicates that no lesions were found in the glands.

bacterial contamination. The gland was then washed free of glycerol, emulsified, and used for injection into the next group of guinea pigs. In some cases, both the submaxillary gland and the parotid gland were examined histologically to see if the infection had spread from the submaxillary gland to the parotid.

None of the right submaxillary glands removed before the inoculation of the left submaxillary gland showed the specific lesion. The great majority of the injected glands showed acidophilic nuclear inclusion bodies and were virulent when inoculated into the brains of young guinea pigs. In one instance, both the submaxillary gland and the parotid glands showed the specific lesion, in four others the nuclear alterations were found in the submaxillary gland only.

Summary of Transmission Experiments.—The experiments recorded in Tables III to V indicate fairly conclusively that the guinea pig virus can be transmitted in series. The striking characteristic of this virus is its marked predilection for glandular tissue, which accounts for the fact that it was impossible to transmit the virus from brain to brain.

Is the Guinea Pig Virus Related to "Kurloff Bodies"?—The occurrence of peculiar structures, known as "Kurloff bodies," in the cytoplasm of the mononuclear cells of guinea pigs' blood was first observed by Kurloff (3) in 1888. Although many investigators have studied the "Kurloff bodies," it has been impossible to draw any definite conclusions as to their nature. Some workers maintain that they represent vacuoles, others that they are true inclusion bodies belonging to the "Chlamydozoa." Their infectious nature, however, has never been proved. All attempts to transmit them from one animal to another have failed. It is of interest that "Kurloff bodies" are not present in the blood of guinea pigs at birth. According to Senez (4) they appear as early as the 7th day; Bender (5), on the other hand, has never observed them in guinea pigs less than 1 month old. The absence of "Kurloff bodies" in very young guinea pigs and the development of these structures in the blood of the majority of full grown guinea pigs, suggested that there might be some relationship between them and the infection of young guinea pigs with guinea pig virus. The fact, however, that "Kurloff bodies" always lie in the cytoplasm of the mononuclear cells, whereas the lesion of the guinea pig virus is characterized by nuclear inclusion bodies, indicates that there

is probably no relation between the two conditions. In order to exclude this possibility, citrated blood of a full grown guinea pig containing "Kurloff bodies," was injected intracerebrally into a young guinea pig. The animal remained well. On the 6th day it was killed and sections prepared from the brain. No meningitic exudate or other cerebral lesion was found. In order to obtain a greater concentration of mononuclear cells containing "Kurloff bodies," 10 cc. of sterile broth was inoculated into the peritoneal cavity of a full grown guinea pig. The following day the peritoneal exudate was examined for the presence of cells containing "Kurloff bodies." A fair number of these cells were found to be present. The cells were washed with salt solution and injected intracerebrally into two young guinea pigs. These animals also remained well.

CONCLUSIONS.

1. It has been shown that the guinea pig virus localizes in the submaxillary glands of young guinea pigs following subcutaneous, intraperitoneal, or intravenous injection of active material, and that the specific lesion is demonstrable in the glands in 12 to 15 days. When an active infection of the gland has been produced in this way, the guinea pigs are refractory to intracerebral inoculation of the virus.

2. No lesion develops in the submaxillary glands of young guinea pigs injected subcutaneously with guinea pig virus which has been inactivated by heat. Young guinea pigs which have received injections of heat-killed virus do not become refractory to intracerebral inoculation of the virus.

3. When young guinea pigs from which both submaxillary glands have been removed are injected subcutaneously with active virus, the virus localizes in the parotid gland, and the animals become refractory to intracerebral inoculation.

4. It has been impossible to demonstrate virucidal properties in the sera of adult guinea pigs which have become spontaneously infected with the virus, or in the sera of young guinea pigs which have been artificially rendered refractory to intracerebral inoculation.

5. It has been possible to transmit the virus from guinea pig to guinea pig continuously in series through seven animals by direct inoculation from submaxillary gland to submaxillary gland.

6. The fact that the virus regularly localizes in the submaxillary glands following subcutaneous inoculation has been utilized in passing the virus from guinea pig to guinea pig. 2 weeks after the subcutaneous inoculation of the virus into young guinea pigs, the active agent was present in the submaxillary glands. Emulsions of the submaxillary glands of these animals were then used for the subcutaneous injection of another group of young guinea pigs. In this way the virus was transmitted continuously from skin to submaxillary gland through a series of seven animals.

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STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

IV. EFFECTS OF CERTAIN PHYSICAL AND MECHANICAL FACTORS ON EXTRACORPOREAL THROMBOSIS WITH AND WITHOUT THE USE OF ANTICOAGULANTS.

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It is well known that coagulation of drawn blood may be retarded experimentally by cold, and that moderate heating hastens coagulation. In fact, it has been claimed by some (Starling) that platelets increase in numbers in cold blood and that they can actually be "manufactured" by cooling platelet-free plasma. In this connection I have no evidence to offer, but it is important to know the influence of heat and cold on clot formation in the circulating blood. This may have some bearing on matters of treatment; consequently some experiments have been carried out by the method already described for the study of extracorporeal thrombosis.

Effects of Temperature.

Cold.—Rabbits weighing about 2 kilos were anesthetized and operated on as already described. The apparatus, filled with physiologic solution of sodium chloride was attached to the carotid artery and jugular vein, the collodion tube being immersed in cool physiologic sodium chloride solution (5°C.). The clamps were detached and the blood began to circulate very violently through the apparatus. In spite of the container being surrounded by ice, the cool solution in the container became warm (10° to 15°) very soon, as it was influenced by the warm arterial blood. To keep the collodion tube cool the solution in the container was changed many times.

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On marked cooling, the blood in the apparatus continued to flow for from 15 to 25 minutes, while at body temperature it ceased in from 4 to 10 minutes. Finally the circulation was stopped by the obstruction of the arterial cannula (rarely of the venous cannula) by white thrombi. Before obstruction was complete the blood current became slow and white thrombi appeared in several places in the apparatus, especially in the arterial tubes and to some extent in the collodion tube. Still circulation sometimes went on. Gradually the pulsation of the collodion tube and jugular vein became less marked, and mixed thrombi appeared in the venous cannula, and extended into the vein. When the apparatus was detached, as soon as the obstruction occurred, the blood in the arterial cannula and in the arterial half of the apparatus was already clotted and mixed thrombi appeared on the irregular surface of the connecting parts of the glass tubes, while about half of the blood in the venous half of the apparatus still remained fluid. In the collodion tube the blood was clotted in laminæ, a part of the blood still remaining fluid; a few small white thrombi were sometimes seen also.

From this it is clear that low temperature tends to retard clotting in the artificial loop and consequently the occurrence of obstruction is postponed. Nevertheless, white thrombi were observed in several parts of the apparatus.

Heat.—In two experiments the effects of heat (40°C.) were investigated. After a few minutes circulation, fibrin formed on the abnormal surface of the apparatus, especially in the collodion tube and venous cannula. Obstruction of the venous cannula by red clot occurred after from 4 to 6 minutes circulation. Soon after obstruction, all the blood in the apparatus clotted as a solid column; lamination was not marked. The formation of white thrombi was scarcely evident.

The results of heat (55°C.) applied around the collodion tube are much more interesting for they indicate the rapidity with which clotting may occur. After from 2 to 6 minutes the venous cannula was totally obstructed and a solid column of blood was taken out of the apparatus. In serial sections only coagulated blood was seen, instead of the characteristic structure of red and white thrombi. Rapid coagulation does not allow sufficient time for stratification or collection of platelets.

Effect of Mechanical Factors Such as Compression and Obstruction of Vessels.

When the blood is normally coagulable and the carotid artery is partially compressed and the stream retarded the arterial cannula is soon obstructed by white thrombi, mixed thrombi form in the artery, and the entire blood in the apparatus comes to a standstill and quickly clots as a solid mass.

Partial Occlusion of the Carotid Artery.—The results in the following protocol are typical of the findings in three experiments.

At 2.25 p.m. Rabbit 28, weighing 1,800 gm. was injected intravenously with 30 mg. of heparin. At the same time the apparatus was attached and the carotid artery compressed with metal clip so that the blood flowed at the rate of 30 drops a minute. Clotting time was more than 30 minutes.

At 2.55 pulsation was recognizable. A white thrombus obstructing the arterial cannula was removed. With a new set-up, the experiment was continued, the blood flowing at the rate of 30 drops a minute.

At 3.20 the stream stopped again. The collodion tube was cut off. The constricted part of the arterial cannula was filled with white thrombi and totally obstructed. There was a big red thrombus (6 mm. long, 4 mm. wide and 3 mm. thick) in the bottom of the arterial end of the collodion tube, and in the arterial half of the apparatus were many white thrombi, which were covered by sedimented erythrocytes.

In spite of the incoagulability of the blood, the slowing of the blood stream has a tendency to facilitate the formation of red thrombi on the foreign lining of the apparatus and, on account of the marked retardation of the stream, the thrombi are chiefly formed in the arterial half, in the collodion tube at the arterial inlet just as in the case of Aschoff's sand banks. Obstruction of the arterial cannula by a mass of white thrombi is easily demonstrated.

In the serial sections this thrombus from the collodion tube is built on a base of very tiny white thrombi and is composed of red clots, in which wide fibrin bands are interwoven. Despite the incoagulability erythrocytes easily precipitate and fibrin is formed rather quickly.

Complete Obstruction of the Carotid Artery.—In two experiments with anticoagulant injection* a few minutes after the circulation through the apparatus was established, the carotid artery was obstructed by a clamp. The apparatus was detached 15 minutes and 50 minutes respectively, afterwards. There was a mixed thrombus

* With larger or continuous dose of heparin clotting might be entirely prevented.

in the carotid artery between clamp and cannula which followed the white thrombus of the constricted part of the arterial cannula. In the latter, also in the venous cannula, in the jugular vein and here and there in the other parts of the apparatus, blood clot adhered to the wall. In the collodion tube the greater part of the blood remained fluid but, in the bottom, thin layers of red clots and one big mixed thrombus adhered to the wall of the arterial half of the collodion tube near the inlet and another was present at the outlet.

In the case of partial obstruction of the carotid artery the blood stream in the apparatus is at a standstill and further growth of white thrombi is very much reduced because no new blood platelets or leucocytes will be brought in contact with the wall.

Obstruction of the Jugular Vein.—The findings in the following are characteristic of those in four experiments.

At 2.30 p.m. Rabbit 44, weighing 1,850 gm. was injected with 40 mg. of heparin intravenously. At 5.05 the apparatus was attached and marked stream and pulsation were observed in it. At 5.15 the jugular vein was obstructed by a clamp. Then the blood stream in the arterial cannula was violent and the pulsation of the collodion tube still marked; soon afterwards the blood in the venous tubes and in the collodion tube became dark, but the pulsation in the arterial half persisted.

At 5.45, 55 minutes after connection, the clotting time was longer than 30 minutes; pulsating movement in the arterial cannula was still marked. 1 hour after the connection the apparatus was detached. In the apparatus white thrombi were not numerous. Sedimentation of erythrocytes occurred in the venous corner tube. The collodion tube contained at its arterial end a large mixed thrombus, consisting mainly of large clumps of platelet thrombi; about twenty clumps were connected by fibrin and erythrocytes.

SUMMARY.

The effects of environmental temperature on thrombosis in circulating blood were tested in the extracorporeal loop by the method previously described. Cold (7° to $15^{\circ}\text{C}.$) about the collodion tube retards the clotting of circulating blood. Obstruction of the arterial cannula occurs after from 15 to 25 minutes. Heat (40° and $55^{\circ}\text{C}.$) tends to hasten clotting; obstruction by red clot takes place after from 4 to 6 minutes and from 2 to 4 minutes, respectively. In the latter case no characteristic thrombus structure may be seen. These results were what might be expected. The formation of white thrombi

occurs even under the influence of cold, and they continue to form and grow as long as the circulation continues.

When the carotid artery is partially occluded and the blood stream slowed, after the injection of the anticoagulant, the blood has a tendency to form red thrombi on the foreign surface of the vascular loop. The thrombi are deposited chiefly in the arterial half of the apparatus, especially at the bottom of the arterial end of the collodion tube, just as in the case of Aschoff's sand experiments. In spite of incoagulability of heparinized blood, red clots, with interwoven fibrin bands, are found. Also very tiny white thrombi may form in other parts of the collodion tube.

The effect of complete obstruction of the carotid artery and jugular vein on thrombus formation was studied after the use of anticoagulant. When the carotid artery is obstructed the formation of white thrombi is negligible and sedimentation of erythrocytes and deposition of fibrin appear throughout the apparatus; then the blood, at a standstill, clots very slowly.

When the jugular vein is obstructed, dislodgment of platelet thrombi probably results from the pulsating movement, and sedimentation of erythrocytes follows, forming mixed thrombi. The dislodged white thrombi tend to gather in the bottom of the tubes of the widest caliber, especially in the collodion tube.

STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

V. INFLUENCE OF CERTAIN CHEMICAL SUBSTANCES ON EXTRACORPOREAL THROMBOSIS WITH SPECIAL REFERENCE TO THE EFFICACY OF A COMBINATION OF HEPARIN AND MAGNESIUM SULFATE.

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With the method of studying experimental extracorporeal thrombosis already described, the influences of certain chemical substances, such as distilled water, bile salts, *d*-glucose, urea and calcium chloride, have been examined.

The Effect of Distilled Water.

It is well known that shed or hemolyzed blood, when injected into the circulation, may cause the liberation of fibrin and subsequently intravascular clotting. On the other hand, it has been stated by Reed that heparinized blood, despite its incoagulability, can clot very soon after the addition of distilled water. In this connection, I have carried out a series of experiments and have verified this fact, as will be seen from the results.

Experiment 1.—A rabbit weighing 2100 gm. was given 50 mg. of heparin (Hynson, Westcott and Dunning) intravenously. Blood was drawn immediately from the carotid artery into a paraffined vessel; 1 cc. of the blood was put into each of a series of small test-tubes and various amounts of distilled water were added to each and clotting time was recorded (Table I). Accordingly I attempted to see what the effect would be of distilled water placed outside of the collodion membrane.

Experiment 2.—When the collodion tube of the circulatory loop was surrounded by distilled water, the latter passed through the membrane into the circulation, causing, from 5 to 10 minutes later, hemolysis of the heparinized blood flowing along the wall of the collodion tube. 30 minutes or more later, fibrin formed, and

coagulation of hemolyzed blood followed. In the serial sections this clot looked almost entirely homogeneous, and neither structure nor formed elements could be differentiated because of hemolysis.*

Two reasons are advanced to explain this phenomenon: one that tissue extract or fibrinogen and hemoglobin set free by hemolysis neutralize the heparin action, and the other, dilution of the anticoagulant. According to Howell, the latter explanation is less probable.

TABLE I.

Clotting Time of Heparinized Blood in Water.

	5 min.	7 min.	12 min.	20 min.	40 min.	60 min.	18 hrs.
1 cc. of heparinized blood in 0.1 cc. water	—	—	—	+	+	++	++
1 cc. of heparinized blood in 0.2 cc. water	±	+	+	+	+	++	++
1 cc. of heparinized blood in 0.5 cc. water	+	+	+	++	+++	+++	+++
1 cc. of heparinized blood in 1.0 cc. water	+	+	+	++	+++	+++	+++
1 cc. of heparinized blood in 2.0 cc. water	—	—	—	+	+	+	++
1 cc. of heparinized blood in 3.0 cc. water	—	—	—	—	—	±	±
Control.....	—	—	—	—	—	—	—

The Influence of Bile Salts on Clotting in the Heparinized Animal.

For a long time it has been known that jaundice tends to disturb the clotting process of the blood. This fact was also verified by Minot and his associates, who found, by using Howell's recalcification method, that the coagulation time was increased in a series of cases of jaundice. Haessler and Stebbins demonstrated that the clotting time of the blood, to which bile salts have been added, is proportional to the quantity of bile salts present and that the bile interferes with the conversion of fibrinogen into fibrin and not with the formation of

* It will have been observed that the tenacity of both red and white thrombi has varied under different experimental conditions. This may depend on variation in the character of the agglutination of the elements or on the amount and nature of the fibrin deposited. Since thrombi *in vivo*, under different conditions, show similar variation in tenacity, the relation of these phenomena to embolism assumes prime importance. Pathologically the question is not only why thrombi form, but why emboli are dislodged in some cases and not in others. In this connection it is to be remembered that fibrin deposited from fibrinogen *in vitro* by any other than biologic methods is not crystalline (Howell).

thrombi. To my knowledge, no one has yet investigated *in vitro* or *in vivo* the influences of bile salts on heparinized blood. I carried out some experiments with a rabbit, whose blood had been rendered less coagulable by intravenous injections of heparin, also some *in vitro* with the blood of normal rabbits, to which heparin was added (16 mg. to each 40 cc.) and found that in both instances more than 1 drop of 1 per cent solution of bile salts to each cc. of blood caused hemolysis, but no decrease of the clotting time.

Experiments were carried out by means of the extracorporeal loop after the injection of heparin. Bile salts, dissolved in physiologic sodium chloride solution (1 or 2 gm. for each 100 cc.) were placed in the container surrounding the collodion tube of the apparatus, and also, in other cases, introduced directly into the circulatory loop through the rubber tube connecting the arterial cannula and the side tube. Thus the simultaneous influence of bile salts and of heparin on experimental thrombosis was observed.

In the former case, that is with bile salts in the dialyzing container, so long as no coagulation or no obstruction occurred the white thrombi not only appeared in abundance in the venous half of the apparatus (the glass tube, paraffined part and collodion tube) but they grew more rapidly than when physiologic sodium chloride solution was used. Radiation, or fan formation, was less pronounced. In the structure of this thrombus, the platelets, of course, play a great part, but the rôle of the leucocytes as a constituent of the thrombus should not be overlooked. The size of each white thrombus at the end of 1 hour or 3 hours was many times larger than when physiologic sodium chloride solution was used for a corresponding period. They appeared as cauliflower-like excrescences.

The thrombi obtained after injection of bile salts were also unquestionably larger than when physiologic sodium chloride solution alone was used. Furthermore, the white thrombi appeared in the arterial half of the apparatus, especially near the place of injection, in the arterial corner tube and in the arterial end of the collodion tube, despite the speed of the stream. The arterial corner tube was nearly obstructed after 3 hours, in spite of the fact that its lumen was many times greater than that of the narrow part of the cannula.

In both cases, when the solution of bile salts was placed outside of

the collodion tube and also when it was injected into the circulation, the mass of white thrombi was loose, soft and fragile, being easily broken up into the individual platelet thrombi. There appeared to be less contractility, suggesting that fibrin was absent or if present was abnormal or of a poor quality. Even when complete obstruction

TABLE II.

Size and Number of White Thrombi with 1 or 2 Per Ccn! Bile Salts Placed Outside of the Collodion Tube.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			mm.	mm.		
Mar. 22	9	3 hrs.	0.60 0.50	0.50 0.50	2 3	No obstruction
May 6	22	3 hrs.	1.40 0.50 0.08	1.20 0.40 0.10	2 5 7	
			0.40 0.20	0.20 0.20	8 5	
May 13	25	4 hrs., 20 min.	1.40		Many	Long ranges of hillocks. No obstruction because the white thrombi gathered mostly in the wide part of the apparatus in the corner tube
June 28	52a	3 hrs.	0.10 0.20 1.0	0.08 0.20 0.8	More than 20	Two ranges of hillocks
June 28	52b	2 hrs., 30 min.	3.0 0.06	4.0 0.08		Large white thrombus in the corner tube obstruction

by a thrombotic mass occurred, no clotting followed. These phenomena are in part due to the action of the heparin, but it seems reasonable to consider that the bile salts also disturb the coagulation of heparinized blood, since such extreme results were not found with heparin alone.

The fact that white thrombi are readily stimulated by bile salts and the red thrombi formed through the influence of bile salts are fragile, might explain why postoperative pulmonary embolism is found in a relatively large number of cases of hepatic disease and gall stones (Wilson).

Typical protocols from a series of experiments follow, and the results of the series are shown in Tables II and III.

Experiment 3 (Bile Salts by Dialysis).—Rabbit 52a weighing 1800 gm. was injected intravenously with 40 mg. of heparin at 12.20 p.m. At 12.40 p.m. the apparatus was connected and 2 per cent bile salts in physiologic sodium chloride solution put outside the collodion tube. The blood stream was very rapid, pulsa-

TABLE III.

Size and Number of Thrombi with 2 Per Cent Solution Bile Salts Injected into the Vessel.

Date	No.	Time	Length	Width	No. in one field
1926		hrs.	mm.	mm.	
June 1	39a	3	1.00	0.80	3
			0.54	0.50	2
			0.40	0.40	3
June 1	39b	3	0.56	0.40	3
			0.44	0.40	3

tion of the collodion tube and the jugular vein being marked. The clotting time was more than 30 minutes. At 3.40 p.m. the collodion tube was removed. Throughout the whole apparatus many rather large white thrombi were seen, most of them confluent and constituting an inner coat or cast on the inner surface of the tubes. The individual thrombi were connected by abnormal appearing fibrin threads. In the collodion tube, similar conditions prevailed. There were innumerable small thrombi but a few were very large. The smaller types fused together to form the thick membrane composed of platelet clumps with numerous leucocytes. In one field more than twenty of the white thrombi measured 0.10 by 0.08 mm., and seven measured 0.20 by 0.20 mm. The large variety were many times larger than those observed in the case of physiologic sodium chloride solution in the heparinized animal. Microscopically large high hillocks of platelet clumps were seen with numerous leucocytes in them. The hillocks measured 1.0 by 0.8 mm., with sometimes a range of hillocks of 0.5 mm. width with valleys intervening.

*Experiment 4 (Bile Salts Injected into the Arterial Side of the Apparatus).—*Rabbit 39a weighing 1020 gm. was given 40 mg. of heparin intravenously at 11.30 a.m. At 12.00 the apparatus was connected and 3 cc. of 1 per cent solution of bile salts was injected into the arterial side of the extracorporeal loop. At 1.00 p.m. 1 cc. of solution of bile salts was injected in the same way. There was a violent stream and marked pulsation in the artificial vascular loop. At 2.00 p.m. 1 cc. of solution of bile salts was injected. At 3.00 p.m. the apparatus was detached; the blood in it remained incoagulable. In the arterial corner tube there were many white thrombi which obstructed the lumen. In the arterial end of the col-

TABLE IV.

Size and Number of White Thrombi with 1 or 2 Per Cent d-Glucose Solution Outside of the Collodion Tube.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			mm.	mm.		
May 7	23b	1 hr.	0.10 0.04	0.08 0.04	More than 20 More than 25	In the figure of radiation
June 21	48a	3 hrs.	0.26	0.024	4	
June 21	48b	3 hrs.	0.20 0.40 0.20	0.40 0.20	Many	Irregular ranges of hillocks Radiation
June 21	48c	2 hrs.	0.10	0.10	12	Radiation
July 19	61a	2 hrs., 30 min.	0.24 0.12	0.10 0.10	6 5	
July 19	61b	30 min.				Blood coagulable
July 21	65	2 hrs.	0.16 0.10	0.16 0.08	6 4	Radiation

lodon tube a good many large white thrombi had gathered, almost obstructing it. Also, in the outer lower part and bottom of the collodion tube there were numerous rather large white thrombi, each hillock being high. Fern-like radiating figures were also seen. In one field, two or three white thrombi measured 1.00 by 0.88 mm., two measured 0.54 by 0.50 mm. and three measured 0.40 by 0.40 mm.

Influence of d-Glucose.

Changes in blood sugar content may cause only slight fluctuations in the clotting time of the blood. It was desirable to investigate the

influence of *d*-glucose on thrombosis, because there is a high blood sugar content, not only in diabetes, but also after *d*-glucose has been introduced as a therapeutic measure. The essential data from twelve experiments on the effect of *d*-glucose on heparinized blood *in vivo* are presented. When *d*-glucose dissolved in physiologic sodium chloride solution (1 or 2 gm. for each 100 cc.) was placed in the container outside of the collodion sac, numerous tiny white thrombi appeared in the course of time (Table IV). The size of each at any given time was relatively smaller than those seen with physiologic sodium chloride solution in the container. The thrombi in the

TABLE V.

Size and Number of Thrombi with 10 Per Cent Glucose Injected into the Vessel.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			mm.	mm.		
June 23	50a	3 hrs.	0.08	0.04	1 or 2	
June 26	51a	3 hrs.	0.08	0.06	9	
June 26	51c	3 hrs.	0.08	0.06	1 or 2	
June 26	51b	30 min.	No thrombi			Obstruction of carotid artery
June 23	50b	1 hr., 5 min.	6.0	3.0		Red cell clumps and clot

collodion tube tended to assume radiating figures in the majority of cases. They were composed chiefly of platelets, and, on and around them, there were in the earlier period a very few leucocytes but in the later stages a considerable number. Even in an experiment of three hours duration obstruction and consequent stopping of the stream was rare, even though, in the constricted part of the cannula, and under certain conditions elsewhere, the thrombi congregated, making rather a loose mass which could be broken easily; fibrin formation seemed to be delayed and abnormal or poor, while with physiologic sodium chloride solution, despite ample action of the heparin, fibrin did appear in rather a short time, and contributed to the obstruction.

Despite these changes, the *d*-glucose did not appear to shorten the clotting time of heparinized blood.

When the *d*-glucose solution (10 per cent) was injected into the artificial circulatory loop through the gum tube between the arterial cannula and side tube, white thrombus formation was rather rare throughout the apparatus (Table V). These findings suggest that hypertonic sugar solution tends to make platelets and leucocytes conglomerate less readily; hence the thrombotic elements do not precipitate readily nor adhere to the vessel wall so that they are carried away. These results were constant for the series of experiments although they are difficult to explain at present.

Influence of Calcium Chloride.

The exact significance of calcium in fibrin formation is still unsettled. Blood from which the calcium has been precipitated by oxalate or citrate will not coagulate, but the addition of calcium salts will promptly cause it to coagulate. As to the influence of calcium on blood clotting, the hypothesis usually accepted is that calcium ions are necessary for the transformation of prothrombin into thrombin. Howell considers that no kinase is necessary, the calcium activating the prothrombin whenever it is not inhibited by heparin. On the other hand, it is known that the presence of calcium in high concentration in the blood prolongs the coagulation time (Freund, Rosenmann and Loewenstein, and others).

Furthermore, Howell and Holt state that no amount of calcium causes clotting of heparinized blood, showing that the heparin does not act by decalcifying the blood. Considering the various rôles of calcium, such as the calcification of vessels, the field of blood clotting, or its wide intravenous use therapeutically, it is of considerable importance to investigate the influence of calcium on extracorporeal thrombosis. Therefore, eight experiments were performed after the preliminary injection of heparin.

Experiment 5.—Rabbit 26 weighing 1950 gm. was given 40 mg. of heparin intravenously at 1.30 p.m. The apparatus was connected at 1.40 p.m. and the collodion tube surrounded with 1 per cent of calcium chloride in physiologic sodium chloride solution, kept at body temperature. The blood stream had been kept vigorous by twice removing the obstruction of the venous cannula. At

TABLE VI.

Size and Number of Thrombi with 1 Per Cent Solution of Calcium Chloride Placed Outside of the Collodion Tube.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			mm.	mm.		
Mar. 11	4a	4 hrs.	Small, white thrombi covered by red clot			Within 30 min. obstruction and sedimentation (4 hrs. and 20 min. after 50 mg. heparin injection); clotting time 5 min.
Mar. 12	5	2 hrs., 10 min.	0.30 0.16	0.24 0.12	5 8	Obstruction one time; removed Fibrin network
Mar. 15	6a	30 min.	0.16 0.10	0.14 0.06	2 15	
Apr. 25	13b	30 min.	0.10 0.08	0.06 0.06	2 3	
May 15	26	3 hrs.	5.00 2.40 1.20 0.40 0.14	1.5 1.20 1.00 0.30 0.12	1 1 4 4 20	Thrombus 1 mm. thick. Venous cannula cleaned up twice as obstruction occurred

TABLE VII.

Size and Number of Thrombi with Hourly Injections of 2 Per Cent Solution of Calcium Chloride.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926		hrs.	mm.	mm.		
June 4	41a	3	1.10 0.16	0.40 0.12	4 15	
June 4	41b	3				All over the apparatus white thrombi causing obstruction, followed by red thrombi. 6 hrs. and 30 min. after 50 mg. heparin (1600 gm. body weight) injection, the clotting time was 5 min., but 20 min. later it was 2 min.

4.40 p.m. the stream was still violent; the clotting time *in vitro* was more than 30 minutes. The apparatus was disconnected. White thrombi in the tubes were very large and numerous (Table VI), especially in the venous half of the apparatus. The venous corner tube was thickly coated. The collodion tube contained large white thrombi in its venous half, while there were very few in the arterial half.

Experiment 6.—Rabbit 41a weighing 1600 gm. was given intravenously 50 mg. of heparin at 11.00 a.m. At 11.20 the whole apparatus was connected, and the collodion tube surrounded with physiologic sodium chloride solution, kept at body temperature; 2 cc. of 2 per cent solution of calcium chloride was injected very slowly into the annular circulation through the rubber tube, which unites the arterial cannula and the arterial side tube; after that 1 cc. of calcium solution was injected every hour in the same way. By 11.40 a.m., from the place of the injection down to the venous corner tube, numerous white thrombi had appeared; the stream was still vigorous. At 2.40 p.m., after 3 hours, the blood remained non-coagulable; the circulation had not been impeded. 3 hours after connection, the apparatus was detached; in the paraffined tubes a goodly number of large white thrombi adhered to the inner wall, making a thick coat (Table VII). Nevertheless, there remained a tiny tunnel through which the blood had been steadily circulating. In the collodion tube, white thrombi were connected with one another irregularly.

When the collodion tube of the apparatus is surrounded by calcium chloride in physiologic sodium chloride solution (1 or 2 gm. calcium chloride to 100 cc. physiologic sodium chloride solution) and at body temperature, the white thrombi appear chiefly in the venous half of the apparatus (the paraffined part of the glass tube and the collodion tube), and as long as the incoagulable blood flows, thrombi grow rather more rapidly than when physiologic sodium chloride solution is in the container; and the narrow part of the venous cannula is easily obstructed by masses of white thrombi, made up of clumps of platelets and leucocytes, interwoven by fibrin threads; the thrombi are not so fragile as when bile salts or *d*-glucose solutions are used. Sedimentation of erythrocytes readily follows, and fibrin seems to form easily. When the obstructing plug of the narrow part is removed as formed, the thrombi in the venous corner tube and collodion sac develop more speedily than in the case of sodium chloride solution and in the course of 2 or 3 hours the lumen of the corner tube which is many times wider than that of the narrow part of the cannula, is nearly obstructed by a thick coat. Otherwise on account of the prompt plugging of the narrow part, a further supply of hematogenic throm-

botic elements is lacking. As a result in the collodion tube, small white thrombi form, which are almost always covered by sedimented erythrocytes. Similar findings are encountered when 2 per cent calcium chloride solution is injected into the circulation.

Effects of Magnesium Sulfate on Extracorporeal Thrombosis.

The foregoing experiments probably shed some light on the problem of thrombosis; however, the ultimate object of these investigations was the prevention of thrombosis, especially of postoperative pulmonary embolism. For this purpose no special prophylactic methods are commonly adopted. On the basis that the slowing of the blood stream probably constitutes one of the most important causes, exercise and movement of the extremities, and massage have been advised by some in order to accelerate the rate of circulation. Similarly, desiccated thyroid has been employed by Walters.

Mason's attempts to protect experimentally against intravascular clotting with heparin are praiseworthy. But, as my numerous experiments show, the formation of white thrombi on the pathologic lining of the vessels was not prevented by a single dose of the heparin employed. The experiments with the injection of hypertonic *d*-glucose solution suggest that the introduction of hypertonic solution into heparinized blood will tend to combat white thrombus formation. Magnesium sulfate also comes into consideration. It has been employed by Fonio in an indirect method of counting blood platelets, to protect against the cohesive properties of the platelets. Some authors believe that there is a close relationship between the earlier stages of thrombosis and agglutinability of platelets. Recently, Hans Schulte investigated the influence of hydrogen ion concentration on agglutination of isolated blood platelets over a period of 12 hours, and reported that it is most marked at a pH of 4.3 to 3.5. He could recognize no influence of sodium chloride and calcium on the process. On the other hand, the results of my studies on experimental thrombosis show that calcium chloride solution seems to stimulate the appearance and development of white thrombi. One might expect some relationship between the presence of calcium chloride in the blood and the development of white thrombi. If, from the colloid-chemical viewpoint, the thrombus formation is closely related to the

agglutination of platelets as colloidal precipitation, magnesium ions and other bivalent positive ions should precipitate the platelets in the same way as calcium does. Thus the influence of magnesium sulfate on thrombosis would appear to be interesting and perhaps of considerable significance. One protocol will suffice.

Experiment 7.—Rabbit 38 weighing 1010 gm., was given 40 mg. of heparin intravenously at 10.25 a.m. At 11.10 a.m. the apparatus was connected and the collodion tube surrounded by 1 per cent magnesium sulfate in physiologic sodium chloride solution. During 3 hours there was a violent stream and marked pulsation in the collodion tube and jugular vein. Clotting time was more than 30 minutes. There was no clotting or obstruction. At 2.10 p.m. the apparatus was detached; there were very few tiny white thrombi in the arterial corner tube. In one place in the upper part of the venous half of the collodion tube an occasional tiny white thrombus was seen, which microscopically consisted of platelets; on each of the thrombi a few scattered leucocytes were recognized. Of twelve thrombi in one field, ten measured 0.08 by 0.04 mm., and two measured 0.10 by 0.06 mm.

From four experiments it was concluded that when the collodion tube was surrounded with 1 per cent solution of magnesium sulfate only a few small white thrombi appeared in the arterial half of the apparatus, while in the venous half very few or no white thrombi were found even when the rate of flow was slow enough to favor adequate formation of thrombi. In the collodion tube, which is the lowest and widest part of the circulatory loop, small white thrombi, often of microscopic size, were seen after 3 hours. The total number of such thrombi was very small in comparison with other experiments. Throughout a 3 hour experiment no obstruction had occurred, neither did any thrombi adhere to the glass cannulas. The action of magnesium sulfate on thrombosis is different from the action of calcium chloride. Magnesium appears to protect definitely against thrombosis, probably by the precipitation of small amounts of calcium as calcium sulfate.

The influence of the intravascular injection of magnesium sulfate solution was next examined. In two experiments the effect of the intravenous injection of magnesium sulfate solution (50 mg. for each kilo of body weight an hour) was tried without the use of any other anticoagulant. The coagulation time of the blood was increased from two to three times normal and thereby clotting in the artificial vascular loop was prevented for from 25 to 30 minutes. White thrombus formation was markedly reduced. Thin layers of blood clot were observed in the collodion tube, and mixed thrombi formed on the irregular surfaces of the apparatus and also in the jugular vein. The venous cannula, and a little later the arterial cannula, were finally obstructed by small masses of white thrombi and fibrin. Despite this, most of the blood in the loop remained fluid.

In the next set of six experiments, the intravenous use of a combination of magnesium sulfate and heparin yielded still more interesting results.

Experiment 8.—Rabbit 7 weighing 2250 gm., was given 20 mg. of heparin intravenously at 3.30 p.m. At the same time 1 cc. of 10 per cent magnesium sulfate was administered intravascularly into the ear vein. At 4.00 p.m. the apparatus was connected and the collodion tube surrounded by physiologic sodium chloride solution of body temperature. At 4.30 p.m. 1 cc. of 10 per cent magnesium sulfate solution was injected intravenously, and at 6.30 p.m. 1 cc. was again injected intravenously. At 7.00 p.m., after 3 hours experiment, the streaming was vigorous; no thrombi were recognized anywhere. The apparatus was detached. In the collodion tube and glass tubes no thrombi were found, even by microscopic examination. The clotting time of the blood was more than 30 minutes.

When a large quantity of 10 per cent magnesium sulfate solution was injected into the annular circulation, or introduced into the general circulation, after intravenous administration of heparin, no thrombi appeared for a certain period in any part of the apparatus. When physiologic sodium chloride solution was used around the outside of the collodion tube, without injection of magnesium solution, white thrombi appeared in 15 minutes in the venous corner tube; 2 cc. of 10 per cent magnesium sulfate solution to 2250 gm. of body weight protected against the occurrence of the first stages of thrombosis for a period longer than 3 hours; 3 cc. of the solution to 2100 gm. of body weight for longer than 6 hours. A few platelet clumps were formed despite the action of magnesium, and sometimes they appeared in the narrow part of the venous cannula, constituting a kind of dam. But when 1 cc. of 10 per cent solution of magnesium sulfate was injected every hour into the circulation of the rabbit, no thrombi appeared. Besides, throughout the experiments, neither obstruction of the circulation nor hindering of the action of heparin has been observed. 3 cc. of 10 per cent solution of magnesium sulfate to 2100 gm. of body weight appears to be almost a lethal dose. This toxicity is at any rate a weak point from the standpoint of clinical application, even though magnesium sulfate solution can prevent the first stages of thrombosis.

Effects of a Combination of Magnesium Sulfate and Bile Salts.—In order to see the antagonistic action between magnesium ions and bile

salts, a series of experiments was performed. When the magnesium sulfate solution, in four experiments, was placed outside of the collodion tube, despite intravascular injection of bile salts, no thrombi appeared in the collodion tube, or if they did, they were scarce and very small. It appears certain that magnesium ions went through the membrane into the circulation, and acted on the platelets and leucocytes in such a manner that the cellular thrombotic elements, passing down along the wall of the sac, were rendered less prone to agglutinate, and were carried away before they were precipitated, also the magnesium sulfate may act to destroy the platelets. When a solution of bile salts surrounded the collodion sac and magnesium sulfate solution was injected into the circulation, the formation of white thrombi was undoubtedly retarded to a certain degree, in regard to number and size, in spite of the stimulating action of bile salts.

This suggests that magnesium sulfate in small amounts will prevent thrombotic elements, especially platelets, from adhering to a pathologically changed vessel wall, partially owing to the platelet-destroying action of magnesium. This may serve to prevent thrombus formation. Possibly the phenomena may be ascribed to changes in surface tension.

CONCLUSIONS.

1. Distilled water dialyzes through the collodion tube and causes hemolysis. Clotting of the hemolyzed blood in the collodion tube occurs later.

2. Bile salts accelerate the appearance and development of white thrombi in the heparinized animal. The masses of white thrombi are very loose, soft, fragile and easily broken into clumps of platelets. They are poor in fibrin or the fibrin is abnormal, since they have less retractility than those obtained from the use of physiologic sodium chloride solution or calcium chloride or serum. Despite the increase in number and size of white thrombi, normal clotting does not occur.

3. When the collodion tube is surrounded by 1 or 2 per cent *d*-glucose solution, tiny and numerous white thrombi appear as radiating figures. The masses of white thrombi are rather loose and fragile. The clotting time of the heparinized blood does not appear to be shortened.

4. When a 1 to 2 per cent solution of calcium chloride is used as the dialyzing fluid outside the collodion tube, or when it is injected into the circulation, the formation of white thrombi is accelerated. They grow very rapidly. In spite of the action of heparin, the white thrombi formed are not so fragile as when bile salts are placed outside of the collodion tube. Fibrin seems to form easily. Obstruction of venous cannula takes place speedily and if the clots in the cannula are not removed, the white thrombi in the collodion tube remain small and become red by sedimentation of red cells.

5. Intravenous use of 10 per cent solution of magnesium sulfate without heparin retards the coagulation of circulating blood and permits the blood to flow through the extracorporeal loop from three to four times as long as normal. The formation of white thrombi, as well as red, is retarded. Magnesium sulfate (1 per cent) in physiologic sodium chloride solution placed outside the collodion tube markedly retards the formation of white and red thrombi in the heparinized animal. Magnesium sulfate (10 per cent), 50 mg. for each kilo of body weight each hour, administered intravascularly in the heparinized animal definitely prevents the first stages of thrombosis, and consequently prevents clotting.

6. It is possible by the combined use of adequate amounts of magnesium sulfate and of heparin intravenously to prevent all stages of thrombus formation for from 1 to 3 hours.

STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

VI. CERTAIN TECHNICAL MODIFICATIONS IN METHODS OF STUDY.

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In the first paper of this series, a method for the study of extracorporeal thrombosis was presented by Rowntree and Shionoya. The technic outlined by them was followed closely by Shionoya in all of his subsequent experiments, as reported in Papers II, III, IV and V. Later the problem was approached from a somewhat different angle, and it was found desirable to modify the original technic. The discussion of such modifications and extensions forms the basis of this report.

Paraffin-Coated Apparatus.

In the original method a portion of the apparatus was coated with melted paraffin, but the cannulas and the rubber collars were left with clean uncoated surfaces. In control experiments with this technic the flow through the apparatus persisted only for from 6 to 10 minutes (rarely 25 minutes if animals were exceptionally large and vigorous), before occlusion of the lumen occurred. It was thought that complete coating of all parts of the apparatus except the collodion membrane might yield results of interest. To this end, the six glass sections and the four rubber collars were assembled and dipped into melted paraffin, before the collodion tube was attached. The paraffin was cooled carefully in order to obtain as smooth an inner lining as possible.

With such preparation of the apparatus, the flow of blood through the loop could be maintained with ease in normal animals for from 2

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to 5 hours instead of from 6 to 10 minutes, a striking demonstration of the important part played by physical variants in thrombosis.

The explanation of this tremendous increase in the duration of flow was not far to seek. With the original technic the glass side tubes were coated with paraffin and then inserted into the uncoated rubber collars until the ends met. If it were possible to secure a water-tight joint by this means such a procedure would be as effective as the coating of the apparatus with paraffin in the assembled state. However, this was not possible, since the ends of the tubes were not ground to fit closely. Thus, when blood passed from one paraffined tube into the next a portion of it came in contact with the surrounding rubber collar and, this being an unparaffined ("water-wettable") surface it facilitated the attachment and disintegration of platelets with the consequent formation of a thrombus at this point. Furthermore,

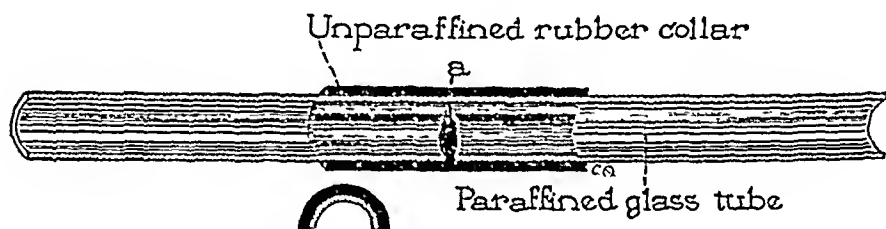


FIG. 1. Sagittal section through joint of extracorporeal loop (early technic).
a, point at which blood came into contact with unparaffined rubber collar.

since at this juncture the lumen of the apparatus expanded suddenly, eddies were formed, thus fulfilling another prerequisite for the formation of thrombi (Fig. 1). When one considers that there were four such points in the original apparatus and that the surfaces of the two cannulas also were clean and unparaffined, it is not difficult to understand how thrombi could obstruct the lumen in one of these areas within 10 minutes in the normal animal.

If one is studying the duration of flow with and without anticoagulant substances (as did Shionoya) the original technic is far better than the modification since it yields more contrast in results and permits the comparative study of duration of flow through the extracorporeal loop. If, on the other hand, one wished to follow, microscopically, the process of thrombosis, the newer method is

preferred since it allows adequate time for detailed study of the early stages of thrombosis.

Size of Collodion Tube.

The collodion tubes used in the first method were 7.5 mm. in diameter and the inside diameter of the glass tubes was but 2.5 mm. Thus, the lumen was suddenly expanded to three times its previous size as the blood left the arterial corner tube and entered the collodion tube. The formation of eddies was marked and the speed of the stream was profoundly decreased. As a result the deposition of platelets and the formation of obstructing thrombi were correspondingly hastened. The large membranes, too, assumed wrinkles of considerable size at points of attachment, another factor predisposing to early thrombosis.

In a later study, tubes just large enough to slip over the ends of the glass tubes were utilized. The pulsation in these is less marked, a factor worthy of attention in microscopic observations, and there is less tendency to early obstruction since the blood current is more rapid and forcible when the diameter of the lumen is decreased.

Direct Microscopic Observation of Thrombus Formation.

A microscope and a carbon arc lamp have been added to the apparatus, making it possible to follow the course of events in the transparent collodion tube throughout the experiment. Using the low power objective as a water immersion lens, the surface flow of blood in the tube can be distinctly seen as well as the gradual formation of white thrombi from their earliest stages. Vital stains have been utilized in this connection. 1 per cent methylene blue introduced into the circulation is taken up by the platelet thrombi, and thus they are outlined clearly against the prevailing red background.

Rapid Method of Permanent Staining of Thrombi on the Collodion Membrane.

As was stated in Paper I, the membranes may be "observed directly, or studied under the microscope with or without the aid of special stains; or the specimen may be prepared and embedded in paraffin for

section staining and ordinary pathologic study." The latter method was used as a routine since it yielded permanent specimens. It possessed, however, outstanding disadvantages; the time element was considerable as the sections went through the busy routine of the pathologic section, and unless there was a considerable mass of thrombi on the membrane the vertical section would show practically nothing. For a study of the early stages of the formation of thrombi on the membrane, this method was wholly inadequate.

To supplement the direct observations through the low power of the microscope, it was desirable to study the membranes with counterstains, and the higher magnifications. It was found difficult and unsatisfactory to do this while the membrane was attached to the apparatus. Attempts were then made to stain the tubes immediately following removal from the loop. After considerable experimentation, a satisfactory method was devised for staining and observing the minute details of early thrombosis on the surface of the membrane. The tube is washed gently in 0.9 per cent sodium chloride solution immediately after its removal from the apparatus and segments about 1 cm. square are clipped out. Some of these small portions are fixed in the vapor of 1 per cent osmic acid. A few drops of dilute ether-alcohol-collodion solution are placed on a clean glass slide and the membrane specimen dried quickly on the outer surface with a blotter or soft cloth. Then before the collodion solution has dried the membrane is placed on it (with the thromboplastic elements uppermost) and gently flattened. The preparation is allowed to dry for a few seconds and is then placed in distilled water for from 2 to 3 minutes. If the membrane does not wash off in the water, it probably will adhere to the slide during the staining process. The slide is then run through a hematoxylin-eosin-alcohol series and finally through carboxylene and xylene exactly as though it were a paraffin section. It is mounted with Canada balsam under a cover slip. The entire process occupies only from 15 to 20 minutes, and several sections may be prepared at the same time.

With this method membranes can be removed from the extracorporeal loop after but a few seconds or minutes of flow and the individual platelets which are attached can be studied long before a tendency toward grouping is manifest. Serial sections are also possible and

thus the gradual growth of a thrombus from the first tiny clump of platelets to the leucocyte, fibrin and red cell stage may be followed and preserved in permanent sections. These sections are really bird's-eye pictures of the progress of events on the surface of the membrane, and supplement adequately the observations made directly with the arc lamp and low power objective during the course of the experiment.

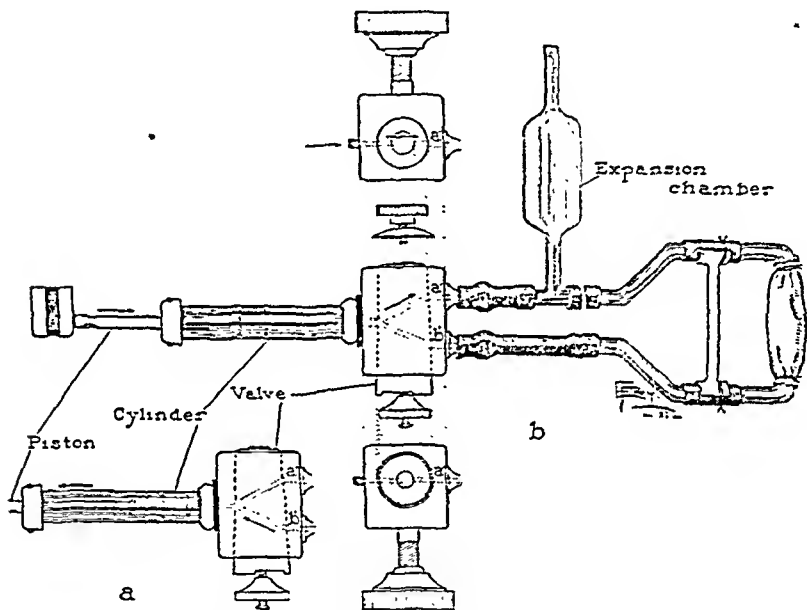


FIG. 2. *a*, piston moving outward (opposite *b*) and current through *b'*, while *a'* is closed by valve. *b*, method of attachment of extracorporeal loop to intake and discharge pipes (*a'* and *b'*) of Woodyatt pump. Piston moving into cylinder—current through *a'* while *b'* is closed by valve.

Artificial Heart.

The extracorporeal loop, minus the cannulas but supplied with an expansion chamber, was attached to the two valves of the Woodyatt pump. All surfaces were carefully coated with paraffin so that at no point would the contained blood come into contact with a water-wettable surface. Whole blood was run directly into the apparatus from a greased cannula in the carotid artery of a rabbit. The motor

was started immediately and a continuous flow of blood through the loop was obtained. Thrombus formation in the collodion tube proceeded at the same rate as in the normal animal, but the thrombi were laid down in longitudinal streaks because of the absence of eddies.

The alternate suction and expulsion of the single cylinder of the pump imparted an intermittent motion (stop-flow-stop-flow) to the blood in the apparatus. The expansion chamber overcame this disadvantage to a certain extent and a fairly continuous flow was finally obtained. Eddy formation being minimal because of the slow speed of the stream, white thrombi (and later, fibrin) were laid down in longitudinal striations, instead of in the usual manner. This is another confirmation of the importance of eddy formation in the growth of thrombi (Fig. 2).

Platelet-containing oxalate plasma was used in the same manner; on recalcification some platelet deposit and fibrin formation was evident. Since the staining method had not been worked out at this time, no microscopic studies were possible.

Additional Modifications.

Instead of the test-tube dialyzing jacket originally used, a small chamber 2.5 by 7.5 by 1.7 cm. made of glass cemented with balsam has been substituted. This chamber is more stable, holds more fluid and permits agitation of contents while dialyzing experiments are carried on. It is also better adapted for use when direct microscopic observations are being made.

In the selection of arterial cannulas, it is well to use the largest one which can be slipped into the vessel. As has been shown elsewhere, the more the cannula is constricted the more easily do obstructing thrombi form.

The medial branch of the external jugular vein of the rabbit possesses a valve system near the point of juncture which prevents reflux of blood into this branch. Consequently when the vein is ligated above this point it promptly collapses and it cannot be refilled except from above. It may be incised and a cannula inserted, without the necessity of clamping, since there is no loss of blood. Thus unnecessary trauma to the delicate intima is averted and the formation of thrombi in the vein practically ceases to be a consideration.

CONCLUSIONS.

As the result of these modifications, many of which in themselves may appear trifling, the course of events in the extracorporeal loop is greatly affected. White thrombi, it is true, are laid down in from 10 to 20 minutes, but the formation of fibrin sufficient to cause obstruction is prevented for 3 hours or more. Thus, the observations in normal animals tend to approach those described in Paper III, after the use of anticoagulants such as heparin and hirudine. The new technic offers certain advantages in the study of the details of the early stages of thrombosis.

THE MECHANISM OF THE INFLAMMATORY PROCESS.*

I. THE ELECTROPHORESIS OF THE BLOOD CELLS OF THE HORSE AND ITS RELATION TO LEUCOCYTE EMIGRATION.

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The present status of the relationship between the electrokinetic potential of leucocytes and the potential differences existing between injured and uninjured tissue zones, points to a situation which is not alone unsolved quantitatively but also qualitatively; but its solution presents, perhaps, a hopeful answer to the question of leucocytic migration in response to injury.

HISTORICAL.

Dineur (1) (1893) seems to have been the first investigator who attempted to study the electrical charge on white blood cells. He placed platinum wires that had been sealed into glass capillaries into the normal and inflamed peritoneal cavities of the frog. Leucocytes tended to enter the capillaries in the absence of a current. In normal frogs, upon the passage of a current, more leucocytes went to the anodal capillary, but just the reverse occurred in frogs with peritoneal exudates. In the latter, the leucocytes seemed to be positively charged, wandering in greater numbers to the cathode. From that time until the present no work has apparently been done which defines *quantitatively* the speed of leucocytic migration and a possible ζ potential. Further, the conditions of the previously undertaken investigations have been so ill defined according to our present knowledge of the behavior of charged colloidal particles, that the investigators' statements seemingly disagree even as to whether leucocytes wander to the anode or to the cathode.

Lillie (2) (1903) studied the white blood cells of the frog suspended in isotonic

* Unless otherwise stated in this and the following papers, the word "inflammation" is used in a strictly limited sense; *i.e.*, the wandering of a white blood cell to a point of injury.

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sugar-NaCl solution. Under these conditions it was found that lymphocytes wandered to the anode; the polymorphonuclear leucocytes to the anode or cathode, or even remained stationary. Höber (3) the following year maintained that frog leucocytes were negatively charged. In 1914, Schwyzer (4), overlooking the definite results of Lillie and Höber, stated that the work of Dineur was untrustworthy, and that his own efforts to produce cataphoresis of leucocytes in tissues were unsuccessful. Schwyzer, however, was the first to hint that electrical differences of potential may play a part in producing the appearances of inflammation. He states: "Der Weg zur Emigration wird durch die Elektronenströme angedeutet, und die Pseudopodien werden durch diese Ströme in der schwachsten Stelle der Membran [capillary wall] gerichtet. Die Leucocytenwanderung im Gewebe wird also *chemotaktisch* aufgefasst, konnte aber auch durch Ionenwirkung erklärt werden." Mendelssohn (5) observed that leucocytes wandered to the cathode. The work done by Höber and his coworkers later leads him to state (6): "Dies hängt damit zusammen, dass nach der Kataphoresegeschwindigkeit und nach der Entladbarkeit mit La^{+++} beurteilt, das ζ potential der Leucocyten nur schwach, negativ, null, oder schwach positiv ist." Eliasoph (7) found that gelatin suspensions of leucocytes wandered to the platinum anode, and that if the electrodes were buried in splenic tissue, there was also anodal migration. Unfortunately, non-polarizable electrodes were not used by Eliasoph. Feringa (8) (1922, 1924) mentions qualitative experiments with rabbit and other white cells. He found them to be negatively charged, but no quantitative data are given concerning speed or potential. Feringa states that lymphocytes do not move as quickly as leucocytes cataphoretically. This will subsequently be shown to be probably a misinterpretation. This author, agreeing with Schwyzer, believed that the potential difference between injured and uninjured tissues caused leucocytic migration to a point of injury, the basis of this taken as pH changes. His observations that leucocytes in general move with ameboid movements toward the anode on an agar floor had not been noted by Abramson (9) on a glass floor for a small potential difference. However, studies on the *floor* of an electrophoresis chamber are complicated by at least the following: (1) the nature of the floor and its influence on the particle itself; and (2) the fact that the suspending medium is also charged relative to the floor. In general, alkaline fluids near the floor of a cataphoresis chamber are positively charged and move toward the cathode. Before one can state, therefore, that ameboid motion is influenced by an electric current, it must be ascertained whether leucocytes would migrate against a moving stream of fluid as occurs in the instance of spermatozoa which move *against* the ciliary stream in the ducts of the female generative organs.

Abramson (9, 10) during the same year (1924) published studies on human lymphocytes suspended in serum. He found human lymphocytes to be definitely negatively charged and estimated the mean

cataphoretic velocity (C.V.) semiquantitatively at 0.3μ per volt per cm. per sec. Further, lymphocytes kept on ice during a period of 30 hours did not appreciably alter the speed of migration. The work on human lymphocytes was undertaken in order to test a hypothesis then proposed which attempted to correlate *quantitatively* the order of magnitude of the potential differences in injured tissues and the speed of white cell cataphoresis (see Fig. 2). The researches of Du Bois-Reymond (11), Biedermann (12), and Hermann (13) pointed to the fact that negative electricity could be conducted from injured tissue surfaces. Potential differences between injured and uninjured surfaces up to about .1 volt had been recorded. With this in mind, it seemed possible that the border of a zone of injury relative to the comparatively uninjured cells, capillary wall, and blood stream could be positively charged. As the surrounding tissue and tissue fluids are conductors, a continuous current must flow between uninjured and injured tissues, the energy coming from the metabolic changes incidental to injury. It was then shown that the order of magnitude of the potential drop between uninjured and injured tissues was probably the same as that necessary to cause white blood cell migration as far as lymphocytes were concerned. That this is also true for polymorphonuclear leucocytes as well will be shown in this communication, and data will be presented concerning the absolute velocities of horse polymorphonuclear leucocytes and lymphocytes in plasma and serum. Observations also have been made concerning cataphoresis in gels and their viscosity. The significance of these data in relation to the inflammatory process will be demonstrated.

Methods.

The method in detail of determination of the C. V. of cell migration is presented elsewhere in connection with the more physical aspects of the present studies (14). In brief, a microscopic method, similar to that used by Northrop (15), was employed which permitted fairly accurate measurement of red cell velocity at different levels in the cataphoresis chamber. From these data the velocity of the medium, V_m , in a given level of the cell could be calculated. The actual velocity of the white cells, V , is then,

$$V = V_o - V_m$$

where V_o is the observed velocity in the particular level. The potential drop in the cell during the measurements was usually of the order of 15 volts per cm.

The Calculation of ζ Potential and the Viscosity of the Suspending Medium.

As is well known (16) the flow of certain colloidal solutions through a capillary, in particular the lyophilic sols, does not follow the Poiseuille formula. That is, with different pressures exerted on the moving column, different values of "viscosity" are obtained. The smaller

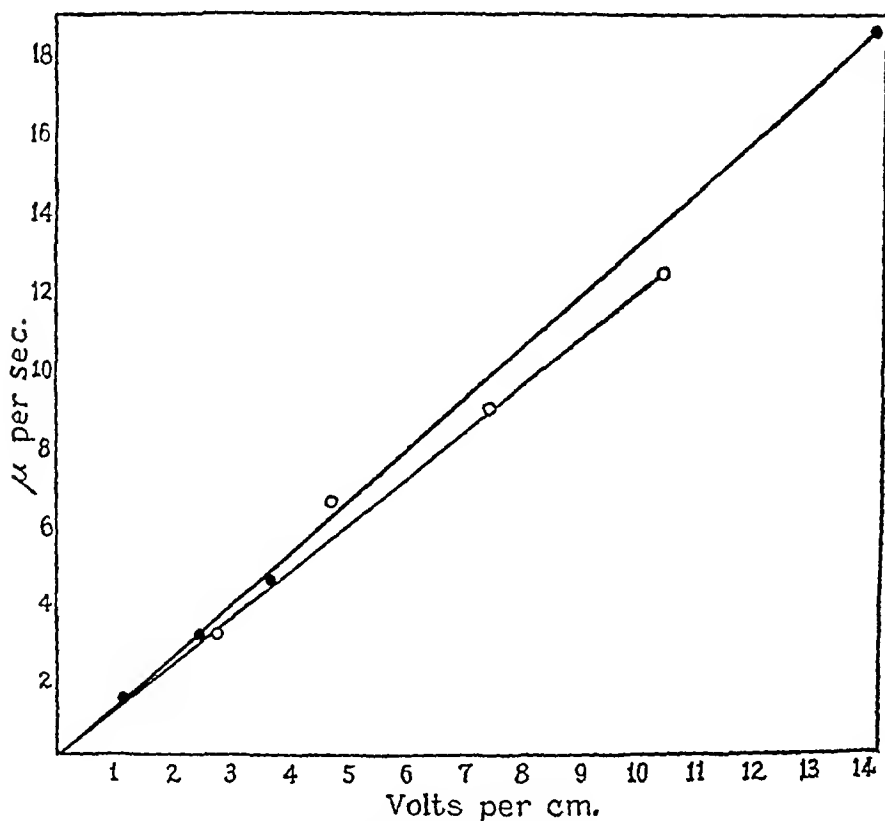


FIG. 1. The C. V. of red cells in plasma is proportional to the applied P. D. The same linear relationship exists for serum.

the pressure, the greater is the "elasticity" of the solution. Blood shows this elastic effect to a slight degree.

From consideration of the Helmholtz-Lamb equation, $v = \frac{HK\zeta}{4\pi\eta}$ (v = velocity, K = dielectric constant of the medium, H = potential drop per cm., ζ = electrokinetic potential, η = viscosity)

(all units are electrostatic), it follows that in a system where the elasticity of the medium could hinder migration, v would not be proportional to H .

Fig. 1 shows that marked differences in H produce a particle velocity which follows the conventional Helmholtz-Lamb equation, and consequently demonstrates that under these conditions plasma and serum have no "elasticity." Accordingly taking the value of .02 for η ,¹ 85 for K (Fürth), and the observed values for the other factors, it follows that for a drop in potential of 1 volt per cm. at 18° the electrokinetic potential

$$= \frac{4 \times 3.14 \times .02 \times (300)^2}{85}$$

$$= (26.5 \times \text{velocity in } \mu \text{ per sec.}) \text{ millivolts.}$$

Evidence has also been obtained by Freundlich and Abramson (14) that the migration of the micellæ of certain "elastic" gelatin gels has the same speed in the gel that is found in the sol. In this instance, a linear relationship between H and C.V. has also been found. This strongly suggests that extremely small differences of potential existing in tissues could produce particle migration even though a viscous gel-like medium was present.

The Electrophoresis of Erythrocytes.

Plasma suspensions of leucocytes and red cells were obtained by oxalating 1 litre of freshly shed horse blood with 8.5 cc. of the saturated solution of the potassium salts. Table I gives the C.V. of red cells in plasma obtained from seven different horses. The mean value .98 μ per volt per cm. per sec. for plasma agrees quite well with the mean value, 1.01 μ per volt per cm. per sec. obtained in serum from ten other horses. The data for serum² are published elsewhere (14).

¹ The viscosity of serum is actually 5-10 per cent lower than that of plasma. This difference is here neglected.

² The data for serum include studies made on aggregates of red cells and red cell rouleaux. It was found that the C. V. of different shaped aggregates were identical with one another and with single cells. The significance of these facts is discussed (14).

Keeping the cells on ice during a period of 3 days or less did not change the C.V. It may be recalled that the isoelectric point of fibrinogen is in the region pH 8.0. Plasma contains .1 to .4 per cent of this protein. The isoelectric point of the other proteins present is in the region pH 5.0. As the isoelectric points of the proteins influence the ζ potential, different C.V. could have been expected from plasma and serum cell suspensions.

TABLE I.

The Electrophoresis of Red Cells in Plasma. Seven Different Horses.

Plasma	Age of cells	Speed	ζ potential
	<i>hrs.</i>	<i>μ per sec. per volt per cm.</i>	<i>millivolts</i>
A	30	1.04	27.5
B	30	1.02	27.0
B		.90	24.0
C	6	1.07	28.0
C serum	6	1.06	28.0
C	30	1.01	27.0
F	6	.97	26.0
F serum	6	.94	25.0
H	6	.91	24.0
I	6	.90	24.0
I		1.01	27.0
J	6	.95	25.0
Mean plasma.....		.98	26.0
Mean serum (10 horses).....		1.01	27.0

The Electrophoresis of White Cells.³

Polymorphonuclear leucocytes (P) wander in plasma (Table II) with a mean C.V. of $.54\mu$ per volt per cm. per sec. (seven horses). This value is about half that found for red cells from the same group

* The C. V. of blood platelets has also been studied and will be presented in a subsequent communication.

(98:54). The calculated mean ζ potential is 14.5 millivolts. Small lymphocytes (SRC) wander in plasma about 20 per cent faster than polymorphonuclear cells. Because of the presence of blood platelets, optimal optical conditions were not present for differentiation of polynuclear cells and round cells. For this reason, serum was studied in greater detail; but it was quite clear that the same relationships held for serum and plasma. In both instances, as found previously for human lymphocytes, keeping the cells on ice up to 48 hours did not alter the C.V.

TABLE II.

The Electrophoresis of White Blood Cells in Plasma (Seven Horses).

(P) = polymorphonuclear leucocyte. (SRC) = small lymphocyte.

Plasma	Age of cells	Corrected speed	ζ potential
	<i>hrs.</i>	<i>μ per volt per cm. per sec.</i>	<i>millivolts</i>
1 (P)	30	.51	13.5
1 (SRC)		.61	16.0
2 (P)	6	.46	12.0
3 (P)	6	.59	15.5
3 (P) serum	6	.50	13.5
3 (P)	30	.57	15.0
4 (P)	6	.57	15.0
5 (P)	6	.47	12.0
5 (SRC)	6	.60	15.5
6 (P)	6	.58	15.5
7 (P)	6	.58	15.5
Mean (P).....		.54	14.5

$$\frac{\text{Red cell}}{P} = \frac{98}{54} = 1.8$$

Table III is a summary of experiments with cells in serum taken from six different horses; the mean velocity of polymorphonuclear leucocytes observed in this series of experiments was $.51\mu$ per volt per cm. per sec. The calculated ζ potential was 13.5 millivolts. Lymphocytes have given a mean velocity of $.60\mu$ per volt per cm. per sec. with a ζ potential of 17.0 millivolts. It is preferred to take the values given in the tables for large round cells (LRC) as approximate because of the difficulties incidental to differentiation of unstained cells.

TABLE III.

The Electrophoresis of White Blood Cells in Serum. Six Different Horses.
(LRC) = large round cell.

Type of cells	Age of cells	Corrected mean (V)	potential	Remarks
		μ per volt per cm. per sec.	millivolts	
1 P	30 hrs.	.50	13.0	P is a mean of 3 values .49, .58, .43 obtained from <i>different</i> levels in cell. These do not agree as well with one another as those given below taken in the midregion.
SRC		.59	15.5	
2 P	6 "	.51	13.5	Although a different chamber than above was used in this experiment, the agreement is excellent P — SRC are mean of 2 experiments.
SRC		.60	17.0	
2a P	30 "	.50	13.0	
SRC		.62	16.5	
LRC		.55	14.5	
3 P	3 days	.61	16.0	In this serum, 3 days old, although higher values have been noted, the ratio P/SRC is practically the same as above.
SRC		.74	14.5	
4 P	2 "	.55	14.5	
SRC		.66	17.5	
5 P	6 hrs.	.47	12.5	
SRC		.54	14.0	
6 P	6 "	.54	14.5	
Mean as above:				
1 P				
2 P				
2a P		.51	13.5	$\frac{\text{SRC}}{\text{P}} = \frac{17.0}{13.5} = 1.25$
4 P				
5 P				
6 P				
1 SRC				
2 SRC				
2a SRC		.60	17.0	$\frac{\text{Red cells}}{\text{P}} = \frac{27}{13.5} = 2.0$
4 SRC				
5 SRC				

TABLE IV.

Typical Protocols of Experiments to Determine Leucocyte Velocity. Horse Serum and Cells.

Column 1, 6 hrs. old. Column 2, 30 hrs. old.

No.	Type	V_0	V	Po- ten- tial		No.	Type	V_0	V	Po- ten- tial	
				milli- volts						milli- volts	
1	P	.73	.48	12.5	Chamber refilled	1	P	.76	.54	14.0	Refilled
2	P	.68	.45	12.0		2	P	.70	.48	12.5	
3	SRC	.87	.62	16.5		3	P	.72	.50	13.5	
4	P	.71	.48	12.5		4	P	.69	.45	12.0	
5	P	.75	.55	15.0		5	SRC	.82	.60	16.0	
6	P	.77	.57	15.0		6	LRC	.79	.55	14.5	
7	P	.77	.57	15.0		7	LRC	.81	.60	16.0	
8	P	.68	.47	12.5		8	SRC	.90	.67	18.0	
9	P	.71	.51	13.5		9	SRC	.76	.56	15.0	
10	SRC	.85	.64	17.0		10	SRC	.97	.72	19.0	
11	LRC	.65	.44	11.5	Refilled	11	LRC	.76	.51	13.5	
12	SRC	1.01	.81	21.5		12	SRC	.82	.61	16.0	
13	P	.69	.49	13.0		13	SRC	.90	.65	17.5	
14	P	.74	.53	14.0		14	P	.63	.47	12.5	
15	P	.67	.46	12.0		15	P	.68	.41	12.5	
16	P	.93	.73	19.0		16	SRC	.76	.61	16.0	
17	SRC	.79	.59	15.5		17	P	.79	.46	12.0	
18	P	.76	.54	14.5		18	P	.74	.52	14.0	
19	P	.68	.48	13.0		19	P	.74	.52	14.0	
20	P	.76	.57	15.0		20	P	.74	.52	14.0	
21	P	.75	.54	14.5		21	P	.69	.47	12.5	
22	SRC	.83	.62	16.5		22	SRC	.79	.54	14.5	
23	P	.70	.49	13.0		23	SRC	.86	.61	16.0	
24	SRC	.90	.69	18.5		24	P	.79	.55	14.5	
25	LRC	.90	.66	17.5		25	LRC	.79	.57	15.0	
26						26	SRC	.84	.63	16.5	
						27	P	.69	.45	12.0	
						28	SRC	.83	.60	16.0	
						29	P	.76	.53	14.0	
						30	P	.76	.53	14.0	
Mean P.....			.51	13.5		Mean P.....		.50	13.0		
Mean SRC.....			.66	17.5		Mean SRC.....		.67	16.5		
						Mean LRC.....		.55	14.5		

The C.V. of single polynuclear cells in serum and plasma was quite constant. In the same preparation (Table IV, Column 1) all but one cell of this type wandered with a speed which varied between .45 and .57 μ per volt per cm. per sec. This variation appears to be much less from *qualitative* observation. Three or four leucocytes accidentally suspended at the same level migrate with the same velocity. The mean value, therefore, of the sixteen cells closely approaches the true value of the absolute velocity for the preparation, .51 μ per volt per cm. per sec. The mean ξ potential, calculated as described heretofore is $(26.5 \times .51)$ 13.5 millivolts.

The behavior of the lymphocytes is as interesting as it is inexplicable. Although a smaller variation in velocity was expected because of the regularly spherical shape, as the table indicates, the velocity of these round cells varies considerably. In general, it may be stated the small lymphocytes wander about 15 to 30 per cent faster than polymorphonuclear cells. The six lymphocytes whose velocities are noted had a mean speed of .66 μ per volt per cm. per sec. The calculated potential is $(26.5 \times .66)$ 17.5 millivolts. Frequently, round cells are observed which migrate more slowly than polymorphonuclear leucocytes. Cell 11, for example, was of the large mononuclear type (LRC). Its migration speed was only .44 μ per volt per cm. per sec. On the other hand, Cell 6, a polymorphonuclear leucocyte, migrated with a velocity of .73 per sec. These differences are all the more striking when observed. The lymphocytes overtake the leucocytes; the red cells go swiftly by with a velocity practically twice as fast; and then suddenly, one may observe a round cell overtaking another round cell, or a leucocyte exhibiting the unusual property of migrating faster than a lymphocyte. This, however, is rare. On the other hand, although potential drops per cm. as high as 30 volts had been used, leucocytes (20° C.) that had settled to the glass exhibited no movement in either direction and could not be removed by a powerful stream of saline forced through the electrophoresis chamber. Feringa's experiments with an agar floor were not repeated because of the questionable influence of water flow on the ameboid movements of the cells mentioned previously. The data, however, given in the tables represent *absolute* velocity within the cells irrespective of the water flow and of ameboid movements.

DISCUSSION.

It has been stated that the present study was a continuation of the work previously done on human lymphocytes in an attempt to correlate *quantitatively* the electrokinetic potential of white blood cells with the differences of potential passing between injured and uninjured tissues (9, 10).

The following discussion is in continuation of this attempt to establish a quantitative basis for this theory of the mechanism of inflammation. It may be again emphasized here that other views, such as those of chemotaxis, of surface tension, etc., are not meant to be excluded. As will be shown, however, there are two quantities: (1) the speed of white cells per volt per cm., per sec.; and (2) the P.D. existing in living injured tissues, whose order of magnitude can be presented with a fair degree of certainty—a degree of certainty which will permit of a correlation of the two, and of a definition in a limited sense of several physicochemical factors incidental to the inflammatory process.

The preceding data lead one to suspect that mammalian white cells are negatively charged and move with an appreciable velocity under a P.D. of 1 volt per cm. toward the anode. With this in mind, the nature and order of magnitude of the P.D. between injured and normal tissues will be described. It is well known that the *surfaces* of injured tissues are negative to the uninjured surface (15). That the uninjured surface of an injured muscle is comparable to the uninjured underlying tissue is shown by the following experiment. If a carefully dissected frog muscle be cross-sectioned, the injured surface at the moment after cutting shows only a small fraction of the maximum E.M.F. which appears only after a considerable interval (17, 11, 13).

Differences in potential arising in tissues incidental to injury cannot have their sources in electrode potentials as no metal is present. Consequently the currents passing between injured and uninjured tissues must have their origins either in: (1) diffusion potentials which are based upon differences in ionic molarities, or (2) membrane potentials, potentials produced by intervening phases—most probably both. From the former, slight electromotive forces are to be expected not greater than .01 volt. On the other hand, membrane potentials are

usually of the order of .02 to .1 volt. Michaelis' discussion of the physiological significance of diffusion potentials does not give them a particularly important position (18). In the instance of an injured tissue zone, the presence of a new phase would, it is true, produce in all

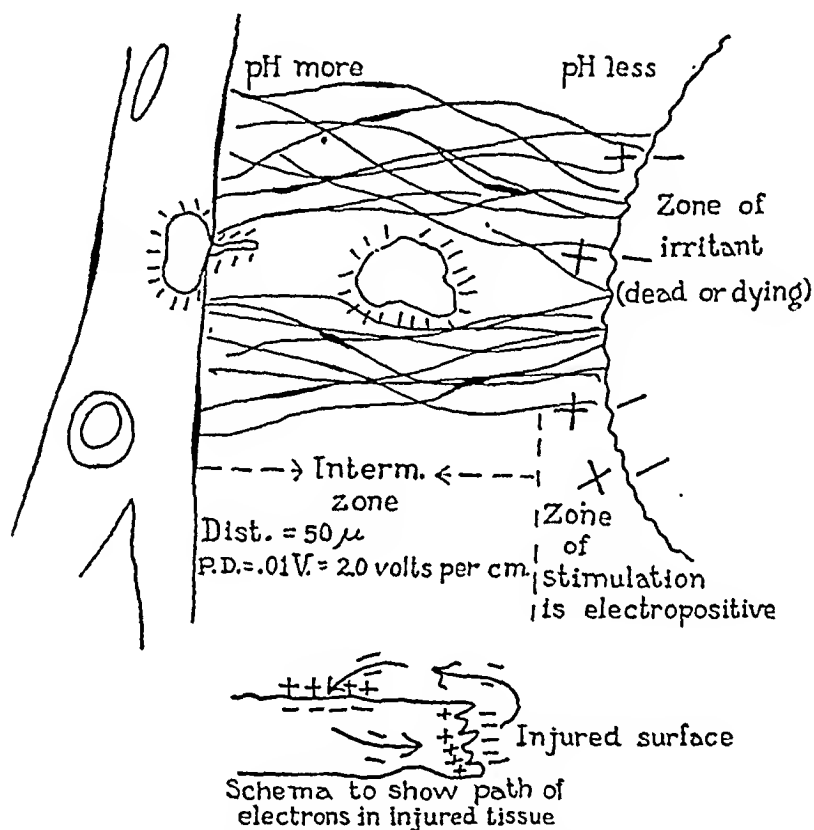


FIG. 2. Note the absence of intervening membranes in the spaces where leucocytic migration occurs between injured area and capillary wall. This structural homogeneity lends aid to the supposition that the conductivity and hence the drop in potential is fairly uniform. For further description see text.

probability potential differences of the order of magnitude possessed by membrane potentials.⁴ The main drop in potential, however, may be across the phase producing the potential. A particle, migrating

⁴ As for example in muscle. A resting muscle is isoelectric. With the production of an injured surface the varying permeability of the membrane of the muscle cells for anions and cations may produce P. D. of the order of magnitude of membrane potentials.

cataphoretically, would thus be out of range of the main drop in potential except when migrating through the phase—*e.g.*, the wall of a capillary. That diffusion potentials may play a significant rôle in the production of cataphoretic migration is shown by the following.

Reference to Fig. 2 shows that negative electricity is drawn from an injured surface. Consequently electrons flow from uninjured tissue zones to injured tissue zones, making the injured area electropositive. That an injured area is relatively positive is made all the more probable by the fact that injured tissues have a lower pH than the blood in the

TABLE V.

This table demonstrates that even with minimum potential differences between injured and uninjured zones appreciable drops in potential per cm. should be found, even with maximum distances.

Distance between injured zone and capillary or normal tissue.....	100 μ	50 μ	25 μ	10 μ
Possible potential differences between injured and uninjured tissue	Drop in potential in tissue			
<i>volts</i>	<i>volts per cm.</i>	<i>volts per cm.</i>	<i>volts per cm.</i>	<i>volts per cm.</i>
.001	.1	.2	.4	1.0
.005	.5	1.0	2.0	5.0
.010	1.0	2.0	4.0	10.0
.050	5.0	10.0	8.0	20.0
.100	10.0	20.0	40.0	100.0

capillaries and adjoining normal tissues (19). Consequently, the higher hydrogen ion concentration (see Fig. 2) again would lead one to believe that injured tissue was electropositive.

Before discussing the order of magnitude of the P.D. existing between injured and uninjured zones, it should be recalled that P.D. is expressed in *volts per cm.* A picture of the forces at play which may influence a charged leucocyte is appreciated only if the drop in potential is converted by estimation of the distance from the injured point to the nearest capillary, to P.D. per cm. Table V shows the estimated order of magnitude of the P.D. per cm. with different distances and different potential differences. The fall across the injured tissue to capillary area would have to be divided inversely proportionally to the resistance of the tissues. As relatively normal tissues have a high resistance

and as the tissue fluids similarly are not good conductors, it is fair to assume that even though the total drop in potential is not uniform, a portion of it, sufficiently great to influence migration of a charged particle, is present. It should be noted that *the inflammatory process takes place in connective tissue*, and leucocytic migration occurs in the capillary spaces between fibrous tissue bands where the P.D. is unhindered by the presence of cell membranes existing in muscle. From the point of view of the structure of the intracellular fluids in connective tissue, one may assume for the present that the thinness and conductivity of phases or membranes present do not make the curve of potential drop sufficiently unlinear to reduce the probability of the correctness of the order of magnitudes to be considered.

Table V shows that even with a P.D. of .001 volt between injured zone and a capillary wall .1 mm. away, the P.D. per cm. is .1 volt. Of course, this is an extreme instance. The potentials measured between cut surface and uninjured surface vary between .030 and .100 volt, and if membranes (phases) are present in the injured zone, this order of magnitude of potential would be expected. If, however, only diffusion potentials incidental to the enormous rise of metabolism which occurs (20) are present and if the P.D. in that instance be only 5 millivolts, with a distance of .05 mm., the P.D. per cm. is 1 volt,—a force sufficient to bring a leucocyte moving in plasma at the rate of $.5\mu$ per volt per cm. per sec. to the point of injury in 2 minutes.

Why do not the red cells and lymphocytes also migrate at once toward the point of injury? Some of them do, but the great difference between these cells and polymorphonuclear leucocytes is that the ameboid cells stick to the capillary walls and are uninfluenced by the rush of blood going by (see Paper II of this series). The speed of blood in capillaries is remarkably constant throughout the vertebrate kingdom. One may expect in mammals a speed of about .5 mm. per second of the cells passing through certain capillaries (21). Even if the P.D. per cm. between blood and injured zone were 20 volts, the speed of a cell moving toward the zone of injury at a rate of $.5\mu$ per volt per cm. per sec. would be only 10μ per sec. Thus, there would be a force exerted on the blood cell in the direction of the blood stream fifty times as great as that exerted in the direction of the point of injury (Fig. 2). When, however, a white cell is trapped by the capillary wall, the electromotive

or other forces are dominant and the forward pressure of the blood stream on the adherent cell is negligible. This presupposed, of course, that leucocytes are able to wander through the wall, as they can. The same phenomena are readily seen in an electrophoresis chamber of the kind described above. At room temperature, on the floor of the cell, are stationary polymorphonuclear leucocytes, and hovering about apparently suspended in the immediate fluid strata are lymphocytes and red cells. Even though the p.d. on occasions has been 30 volts per cm., the leucocytes which have stuck to the glass remain there uninfluenced by the difference of potential while the round cells and red cells move swiftly by just as in a blood vessel.

One further point at present remains to be discussed—the electrophoresis of water. Granting the difference of potential and the other factors that have been previously discussed, it follows that the movement of the tissue fluids would also be influenced. As the experiments of Mudd (22) have conclusively shown, animal membranes are negatively charged when the medium is serum or any other alkaline fluid. The significance of this in relation to the inflammatory process is at once evident. There should be adjacent to the strands of connective tissue and in the pores of the connective tissue a cathodal flow of water. But, these strands of connective tissue form capillary spaces. If these spaces are closed systems, as in an electrophoresis chamber, in the absence of structural irregularities, there may be in the midregions of these capillary spaces a return anodal flow to assist leucocyte migration where the capillary spaces are not too small.

SUMMARY.

1. The velocity of cataphoretic migration of blood cells in plasma and serum is proportional to the potential drop applied.
2. The cataphoretic velocity of red cells, polymorphonuclear leucocytes, small lymphocytes, and large lymphocytes is described for serum and plasma.
3. The relation between the electrokinetic potential of white blood cells and the differences of potential probably existing in injured tissues are correlated quantitatively.
4. This correlation suggests that migration of leucocytes to a point of injury is, in part, dependent upon the electromotive forces at play in the tissues.

The writer wishes to express his thanks to Professor H. Freundlich for his constant guidance and criticisms, and to Dr. Ettisch for his unceasing interest.

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THE MECHANISM OF THE INFLAMMATORY PROCESS.*

II. CONCERNING THE ADHESIVE FORCE OF ONE PSEUDOPOD OF A FROG LEUCOCYTE AND ITS RELATION TO LEUCOCYTE EMIGRATION.

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(Received for publication, July 6, 1927.)

Although much work has been done concerning the adhesive qualities of white blood cells, these studies of stickiness have apparently all been made *in vitro* with glass or other surfaces (1).

This communication deals with observations made on the behavior of a single leucocyte in a capillary of the web of a frog's hind limb. The phenomenon seems not to have been reported. Because of the quantitative considerations which may be deduced from this observation, it is worthy of description in detail. Dr. G. Ettisch was present and confirmed the sequence of events to be described in the following.

1 hour after the frog (*Rana temporaria*) had been anesthetized by urethane, the vascular changes incidental to an inflammatory process produced by a needle-HCl puncture of the web were studied. The vascular channels were wide open with a very rapid flow of blood cells, unhindered except at points where white cells had begun to stick to the capillary walls or roll slowly along the wall. At the bend of a capillary whose width was about four times the diameter of a polymorphonuclear leucocyte, an ameboid cell was seen to be suspended in a rushing blood stream by a single thin white thread of protoplasm (Fig. 1, *a*). The cell was in the centre of the stream, in contradistinction to the other cells along the wall, and was held in place by this thin thread of cytoplasm (about three times as long as the cell itself) which was drawn out to a minute point parallel to the direction of the blood

* See Paper I of this series.

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stream, and was stuck to the wall by an apex whose diameter seemed to be about $1\ \mu$. The preparation as a whole was in the horizontal plane. The cell was definitely seen to be in the middle of the stream surrounded and tossed about by red cells streaming past so rapidly that individual cells could not be observed. The thin thread of cytoplasm (pseudopod) then shortened until the cell approached the capillary wall to about its own length (Fig. 1, *b*).

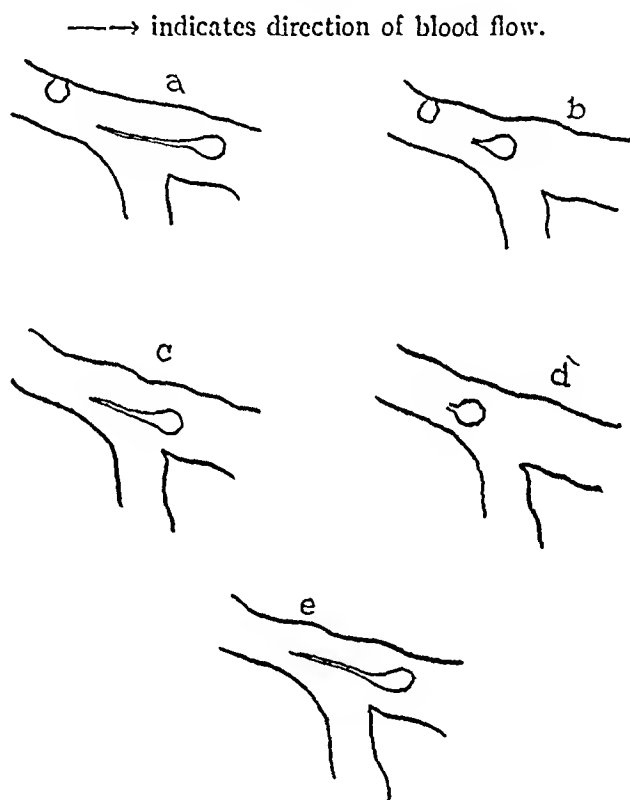


FIG. 1. For description, see text.

Then, seemingly overwhelmed by the force of the blood stream, the pseudopod was again stretched out to its previous length. After several minutes of such play, the pseudopod slowly contracted (Fig. 1, *c*) and the white cell was drawn to the capillary wall (Fig. 1, *d*). This lasted but half a minute when the cell was again drawn out by the force of the blood stream with a thread of cytoplasm as long and as thin as heretofore described (Fig. 1, *e*). After a short period of

tossing about in the centre of the stream, the pseudopod was apparently torn free from the wall and the cell disappeared from the field.

It is evident that the adhesive force exerted by this single pseudopod was equal at least to the resultant of the forces of the stream of blood on the white cell and of the force of gravity. To obtain an idea of the order of magnitude of this force, let it be assumed that the leucocyte was a sphere of radius, r , in a medium whose viscosity, η , was constant and whose speed, v , was also constant. Then, F , the force in dynes, is expressed by the following equation:

$$F = 6 \pi r \eta v$$

Substituting .03 for η , 4×10^{-4} for v , and .05 for r (2), the value of 10^{-5} dynes is obtained for the horizontal component, the force of the blood stream. As the cell was maintained by the blood stream in a horizontal plane, it is evident that the gravitational component was comparatively small. This vertical component is expressed by

$$F = \frac{4}{3} \pi r^3 (D - \delta) g$$

where r is the radius of the cell, D the density of the medium, δ the density of the particle, and g the gravitational constant. It will make little difference what probable value is taken for $(D - \delta)$. Assuming $(D - \delta)$ be equal to 0.1 and substituting,

$$\begin{aligned} F &= \frac{4}{3} \times 3 \times (4 \times 10^{-4})^3 \cdot 10^{-7} \times 10^3 \\ &= 2.5 \times 10^{-8} \text{ dynes.} \end{aligned}$$

It follows then, that the gravitational component is negligible and that the force, 10^{-5} dynes, the "adhesive force," of this single pseudopod was at least of this order of magnitude. Or assuming the area of the pseudopod on the capillary to be 1 sq. μ , the force per sq.mm. would be $(10^{-5} \times 10^6)$ or 10 dynes. This value is so small that the probability of its representing tensile strength rather than adhesive force is slight. The tensile strength of frog muscle, for example, is 6×10^4 dynes per sq.mm. (3).

From the foregoing it may be readily understood why leucocytes stick to a capillary wall with such apparent ease although a seemingly overwhelming force introduced by the blood stream is present. If

this phenomenon be repeatedly observed, it may be accepted that the adhesive force, of the order of magnitude of 10^{-5} dynes, is exerted by one pseudopod. A leucocyte touches the capillary wall with many more pseudopods. And since the force of the stream at the walls of the capillary is less than at the centre, a sufficient resistance may easily be offered by more than one pseudopod to produce leucocyte arrest and preparation for emigration.

SUMMARY AND CONCLUSIONS.

Observations on the behavior of a frog leucocyte *in vivo* have led to the conclusion that an adhesive force of at least 10^{-5} dynes may be offered by one pseudopod. This adhesive force, of one pseudopod, is of the order of magnitude sufficient to cause leucocyte arrest previous to emigration through the capillary wall.

I desire to express my thanks to Professor H. Freundlich for his kindness in reading the manuscript.

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THE STRUCTURE OF *B. ANTHRACIS* AND REVERSAL OF THE GRAM REACTION.*

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PLATES 34 TO 38.

(Received for publication, May 31, 1927.)

If a small amount of aqueous solution of acriviolet, gentian violet, or acriflavine be added to a thick aqueous suspension of a young culture of *B. anthracis* it will be found that after a longer or shorter interval of time a large proportion, if not all, of the organisms are changed by the exposure from sharply Gram-positive to sharply Gram-negative. The speed with which this change takes place varies with the strain of *B. anthracis* examined. In one strain studied, Gram reversal began in a few minutes and was complete within 2 hours. In other strains 19 hours were required to produce the change and even at the end of this period a few Gram-positive individuals were sometimes still found in the smears. The Gram-negative forms of *Bacillus anthracis* produced by exposure to these dyes are notably smaller in calibre than the Gram-positive forms, the difference in diameter as measured by means of a filar micrometer being in the neighborhood of 40 per cent.

That such changes could be produced in this organism by exposure to aniline dyes was first observed about a year ago. The present communication is concerned with studies made, since that time, of the reversal of Gram reaction, and of the change in calibre, which the dyes caused; and with attempts to interpret the significance of the two phenomena.

* Preliminary report of this work appeared in *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 737.

The Gram Stain.

In our early observations as to the effect of aniline dyes on *B. anthracis* the smears were stained by Paltauf's modification of the Gram method.¹ The findings thus obtained were also confirmed by the study of smears stained by the other well known modifications: Claudius',² Burke's,³ Atkin's,⁴ and Kopeloff's.⁵ After the examination of about 2000 smears, each specimen being stained and examined by two observers, the definite conclusion was reached that Burke's modification was superior to all others. It was therefore, from that time on, exclusively used. The opinion as to its superiority was amply confirmed by the subsequent studies which involved the examination of nearly 12,000 smears. Whenever "Gram stain" is mentioned in this report it is the Burke modification (unless otherwise specified) to which reference is made. With the Burke technic the results are constant and absolutely clear-cut. The colors in the final smears contrast sharply (a bluish black with a pink) and one is never left in doubt as to whether an individual organism is to be called Gram-negative or Gram-positive.

When stained by the Burke technic young cultures of *B. anthracis* are definitely and constantly Gram-positive. An occasional Gram-negative individual may be seen, but these are *only* occasional and negative forms are usually entirely absent. The Gram-negative organisms, like *B. coli*, which are commonly used for controls in experimental work, are equally constant and sharp in their reaction to the Burke stain. If therefore the technic as described by Burke be carefully followed and the precautions observed which it is well known must be borne in mind in using the Gram method, the Burke modification is a method of exactness and from its results trustworthy conclusions can be drawn.

¹ Sharnosky, J., *Proc. New York Path. Soc.*, 1909, ix, N. S., 5.

² Claudius, M., *Ann. Inst. Pasteur*, 1897, xi, 332.

³ Burke, V., *J. Bact.*, 1922, vii, 159.

⁴ Atkin, K. N., *J. Bact.*, 1920, iii, 321.

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Exposure of Organisms to Aniline Dyes.

The technic of the experiments in which reversal of the Gram reaction of *B. anthracis* was produced was the following.

A heavy suspension of 4 hour culture of the organism was made in distilled water. $\frac{1}{2}$ cc. of this suspension was placed in each of two tubes. To one of these tubes an oeseful of 1 per cent aqueous acriviolet was added. Smears were made at the beginning of the experiment and stained by Burke's method. These always showed the organism to be 100 per cent positive except that an occasional stray Gram-negative individual might be found. The tubes were put away at room temperature and smears from each tube examined at various intervals. It was found that Gram-negative forms began very soon to appear in the tube to which acriviolet had been added. In the case of the more rapidly changing forms, like No. 10,⁶ reversal of Gram reaction began almost at once and was complete in 2 hours or less (Fig. 1). In the more slowly changing forms like N.Y.B. of H.,⁷ 5 per cent of Gram-negative individuals appeared in $2\frac{1}{2}$ hours.⁸ At the end of 19 hours smears from the acriviolet tube showed that the organism had changed from a predominantly Gram-positive to a predominantly Gram-negative one. In other experiments reversal proceeded until about 75 per cent of the organisms had become Gram-negative and further change occurred very slowly if at all.

The first observations as to the effect of aniline dyes on *B. anthracis* were made with a strain furnished by the New York Board of Health. In order to determine that the reversal phenomenon was not peculiar to this particular strain of *B. anthracis* the experiment was repeated

⁶ The numbers refer to the American Type Culture Collection.

⁷ The strain referred to as "N. Y. B. of H." was isolated at the New York Board of Health Laboratories from a shaving brush in January, 1923.

⁸ The roughness of a method in which percentages of Gram-positive and Gram-negative forms in a smear are estimated without actual counts is of course perfectly understood. Small variations are, under such conditions, of no significance whatever and no attention was paid to them. But the difference between a smear containing 99 per cent positive forms and one containing only 30 per cent positive forms is of course obvious at a glance and it was only differences of this order which were taken into account. In all cases the smears were stained by two observers and each smear recorded by both; the figures of the two records agreed pretty closely. It was found that the changes produced by the dyes were the same even when varying amounts were added and exactness of dilution was not necessary; "1 oeseful of 1 per cent dye" was therefore a sufficiently accurate measure. The oese used measured 2.33 mm. internal diameter.

with Strains 8, 9, 10, 240, 241, and 242 of the American Type Culture Collection. The authenticity of all of these strains was established by animal inoculation. It was found that the reversal phenomenon was produced in each instance by the exposure of the organism to aniline dyes so that the conclusion seemed warranted that the reaction was characteristic for the genus.

Similar experiments were then undertaken with other members of the spore-bearing, aerobic group. The first group studied was *B. subtilis* of which six strains were examined: one strain, isolated from hay, in our own laboratory and Nos. 102, 465, 466, 243, and 3 A. T. C. These experiments with *Bacillus subtilis* showed that a reversal of the Gram reaction similar to that of *B. anthracis* results from exposure to aniline dyes; but the phenomenon was less striking, since in some of the strains a moderate degree of reversal also occurred in the control tubes.

The definite findings in the case of *B. anthracis* and the suggestive findings in the case of *B. subtilis* suggested that the phenomenon might be common to all sporogenous aerobic⁹ bacteria and a study was therefore made of this whole group, the strains being obtained from the American Type Culture Collection. The following organisms were examined.

<i>B. mesentericus</i>	No. 76
" <i>megatherium</i>	" 72
" "	" 71
" <i>mycoides</i>	" 270
" " <i>roseus</i>	" 80
" <i>adhærens</i>	" 271
" <i>niger</i>	
" <i>agri</i>	
" <i>laterosporus</i>	
" <i>simplex</i>	
" <i>globigii</i>	
" <i>albolactus</i>	
" <i>aterrimus</i>	
" <i>panis</i>	
" <i>prausnitzii</i>	
" <i>ruminatus</i>	
" <i>flavus</i>	" 58

⁹ Nothing can at present be said about the behavior of anaerobes.

With a number of these organisms, reversal of Gram positivity was produced just as in the case of *B. anthracis*. This was true for example of *B. megatherium* and *B. mycoides*. But in the case of the majority of strains in this group either the results were inconstant or reversal occurred in the control tubes, so that no statement can be made as to the existence of any general relation between reversal phenomenon and the presence of spores in bacteria.

The investigation was then extended by making a study of non-spore-bearing Gram-positive organisms. Experiments were done with the following 50 strains.

	No.		No.
<i>Sarcina lutea</i>	272 A.T.C.	<i>Actinomyces maduræ</i>	552
" <i>rosea</i>	188	" <i>bovis</i>	549
" <i>aurantiaca</i>	146	" <i>gypsoides</i>	550
" <i>flava</i>	147	<i>Staphylococcus epidermidis</i>	155
<i>Micrococcus auranticus</i>	387	" <i>albus</i>	251
" <i>luteus</i>	379	" <i>citreus</i>	395
" <i>freudenreichii</i>	407	" <i>aureus</i>	477
" <i>flavus</i>	140	" <i>candicans</i>	154
" <i>conglomeratus</i>	401	" <i>tetragenus</i>	159
" <i>flavoreus</i>	397	<i>Bacillus xerosis</i>	373
" <i>cereus</i>	394	" <i>diphtheriæ</i>	475
" <i>varians</i>	399	" <i>hoffmanii</i>	371
" <i>ureæ</i>	408	<i>Staphylococcus pyogenes</i>	160
<i>Rhodococcus ruber</i>	534	<i>Streptococcus lactis</i>	558
" <i>rhodochrous</i>	184	<i>Lactobacillus casei</i>	334
" <i>roseus</i>	185	<i>Staphylococcus</i> (No. 33)	Torrey
" <i>cinnabareus</i>	514	" (Richards)	"
<i>Actinomyces asteroides</i>	322	" (No. 77)	"
" <i>hominis</i>	551	" (isolated by Churchman)	
<i>B. diphtheriæ</i>		N. Y. B. of H.	
" "		" " " " "	
" "		" " " " "	
<i>B. hoffmanii</i>		" " " " "	
Pneumococcus Type 1		" " " " "	
" " 2		" " " " "	
" " 3		" " " " "	
" " 1		Rockefeller Institute	
" " 2		" "	
" " 3		" "	
<i>B. tuberculosis</i> bovine		N. Y. B. of H.	
" " "		" " " " "	

This study of the behavior toward aniline dyes of non-spore-bearing Gram-positive bacteria showed, that in striking contrast to the results produced in the case of *B. anthracis* and other members of the spore-bearing group, the dyes mentioned were without any effect on the Gram positivity of the majority of the non-spore bearers. The experiments with certain of these organisms (about 15 strains) were, however, unsatisfactory and from them no conclusions could be drawn. Pneumococcus for example proved too fragile for this type of experiment and we were unable to get constant results whether we worked with blood agar cultures or with the peritoneal fluid from injected mice. Nor were we able to draw positive conclusions from the experiments with *Streptococcus lactis*, nor with *Actinomyces hominis* or *Actinomyces maduræ*. But for the majority of the organisms (about 35 strains) in this group the dye was absolutely without effect on the Gram reaction, even after many days exposure and even when the tube was heated in order to make the test more severe. The difference between the behavior to aniline dyes of *B. anthracis* and a number of the Gram-positive non-spore bearers was well shown by an experiment in which a suspension containing both *B. anthracis* and *M. freudenreichii* was used. Both of these organisms are definitely Gram-positive and the smears made at the beginning of the experiment showed nothing but Gram-positive forms. Smears made (at the end of 19 hours) from the tube to which acriviolet had been added showed that none of the individuals of *M. freudenreichii* had been changed but that about 95 per cent of the individuals of *B. anthracis* had become Gram-negative. The smears from the control tube at this time showed all the organisms of both strains to be still Gram-positive (see Fig. 2).

It seems clear from the experiments just described, in which the bacterial field was pretty completely covered so far as the Gram-positive aerobic forms are concerned, that two types of organisms may be distinguished, one of which exhibits reversibility of Gram reaction when exposed to acriviolet and the other of which remains Gram-positive in spite of long exposure to the dye. The organisms which exhibit the reversal phenomenon in clear-cut fashion belong in the spore-bearing group; the organisms which resist this effect of the

dye belong in the non-spore-bearing group.¹⁰ This fact is sufficiently striking and may be of significance. There are, however, numerous exceptions in both groups, so that one cannot definitely relate the reversal phenomenon to the presence or absence of sporogenic material.

Other Dyes.

It has been said that the reversal phenomenon is brought about by exposure to acriviolet and this dye did appear to be more effective than any other in producing the result. But exposure to other dyes, notably acriflavine, gentian violet (both Grüber, and Coleman and Bell), and ethyl violet also resulted in a reversal of Gram reaction and a diminution of calibre.

Other Substances than Dyes.

Since the dyes are known to have a bacteriostatic effect upon certain of the organisms which exhibit reversal phenomenon experiments were done with other substances, not dyes, also capable of producing bacteriostasis. In these experiments both *Bacillus anthracis* and *Staphylococcus aureus* were exposed to the action of alcohol, formalin, and Zenker's fluid. These substances produced no effect on the Gram reaction of either organism.

Relation of Reversibility to Acidity.

The dyes used, though basic or neutral in name, have a pH between 3 and 4¹¹ and this fact raises the question also suggested by the work of Deussen¹² (who noted the effect of acids on the Gram reaction—but not on the size—of certain Gram-positive organisms), whether the phenomenon here reported might not be due to the slight acidity

¹⁰ Deussen (Deussen, E., *Z. Hyg. u. Infektionskrankh.*, 1918, lxxxv, 235) noted a similar difference in the effect of certain substances on the Gram reaction of different Gram-positive organisms.

¹¹ The potentiometer readings are as follows:

1 per cent aqueous gentian violet.....	pH 3.5
“ “ “ “ acriflavine.....	pH 3.5

¹² Deussen, E., *Z. Hyg. u. Infektionskrankh.*, 1918, lxxxv, 235. This author regarded the phenomenon as a purely chemical one and thought the effect of acids to be in direct proportion to their degree of dissociation.

(it is very slight) of the bacterial suspension resulting from the addition of the small amount of 1 per cent acriviolet or gentian violet used.¹³ Since the result produced by these dyes was not produced by acid fuchsin (pH 2.55) it seemed unlikely that acidity of dye was the whole explanation of the phenomenon. That the addition of strong acids, at least, did not produce reversal of Gram reaction nor diminution of calibre was easily shown by the following experiment.

Three tubes each containing $\frac{1}{2}$ cc. bacterial suspension (*B. anthracis* N. Y. B. of H.) were prepared. To the first was added 1 oeseful of 1 per cent acriviolet, to the second 1 oeseful of 10 per cent HCl, to the third 1 oeseful of concentrated HCl. Complete reversal took place in the acriviolet tube within 19 hours. In the acid tubes no change was observed at the end of 19 hours, and only slight change at the end of 48 hours.

A second experiment to test this point was done by adding to a series of tubes containing the bacterial suspensions 1 oeseful of water which had been brought to pH 1.2, 1.8, 2.4, 3, 3.8, and 7. In none of these tubes did reversal occur, although complete reversal took place in the control tube containing dye. This experiment is open to the objection that the acid used was not buffered and therefore its action may not have been continuous. In the dyes, on the other hand, acid may be present in just the right proportion and it may be so buffered as to act continuously. Efforts to test this point out by buffering with KCl and with KH phthalate, the acid added to the bacterial suspensions were defeated because it was found that the buffers themselves led to some reversal. The point must, therefore, be left for the present in abeyance.

Modifying Factors.

The question of the relation of the reversal phenomenon to the age of the culture was investigated. This was done by making bacterial suspensions of agar growths, at intervals of 1 hour, from 1 hour up to 7. Though the results of these experiments were not absolutely constant, there is reason for believing that very young cultures are more resistant to the reversing effect of acriviolet than older cultures.

The question of the nature of fluid in which the suspension is made and its relation to the reversal phenomenon was studied by making

¹³ The pH of the distilled water used in this laboratory is 6.0. Potentiometer readings of $\frac{1}{2}$ cc. of this water containing 1 oese 1 per cent acriviolet showed a pH of 5.3+.

suspensions in distilled water, in tap water, and in physiological saline, and adding dye to these tubes. Reversal proceeded more rapidly in the water tubes than in saline.

Reduction in Calibre of Bacteria by Exposure to Dyes.

Reference thus far has been made only to the change in Gram reaction produced by exposure to acriviolet. But another equally striking, if not more striking, change has also been observed; for it is noted in the smears of *B. anthracis* which have been exposed to acriviolet that the Gram-negative forms are much smaller in transverse diameter than the Gram-positive forms. This difference in size is so striking, the organisms stained with safranin being quite slender and those stained with methyl violet quite stout, that one might easily conclude that the smears contained a mixture of two different organisms, one a robust Gram-positive and the other a slender Gram-negative (see Fig. 1, *B*). In smears made when the change is about half complete these pink and bluish black segments may alternate in the same chain giving a most striking appearance (see Fig. 1, *B*, and Fig. 5, *A-D*). It will be noted that when spores are present they are entirely contained within the Gram-negative forms. This is apparent in many of the illustrations; but particular attention is called to the organisms marked *a* Fig. 1, *B*, and *a* Fig. 3, *A*.

Large numbers of measurements of the Gram-positive and Gram-negative forms were made, a Zeiss filar micrometer being used for the purpose. These measurements showed the Gram-positive forms to be practically always larger in transverse diameter than the Gram-negative forms. The difference in size is of course not absolutely constant in degree, varying from $.123\mu$ to $.962\mu$; but in only one instance has a Gram-negative segment of a bacterial chain been seen which was larger than the adjacent Gram-positive segment, and in only the most occasional instances were they of the same calibre. Comparative measurements of 25 Gram-positive and 25 Gram-negative adjacent segments from a smear of *B. anthracis* No. 10 stained by Burke showed: average diameter of the Gram-positive forms 1.2236μ , of the Gram-negative forms, $.7091\mu$, a difference of $.5145\mu$ or $42\pm$ per cent.

In the complete study 292 organisms were measured. The average diameter of the Gram-positive forms was 1.112μ ; of the Gram-negative forms $.686\mu$. This gave an average difference of $.426\mu$ or 38.3 per cent.

When measurements were made of specimens which had been stained by the

Paltauf modification, the same general facts came to light although the difference between the Gram-negative and Gram-positive forms was not quite so marked as in specimens stained by Burke's method (see Fig. 3, *A* and *B*, and Fig. 5, *D*). In this set of observations, measurements were made of 50 organisms. The average transverse diameter of the Gram-negative forms was $.311432\mu$ smaller than that of the Gram-positive forms, a reduction of 29.74 per cent in diameter.

Exposure to acriviolet therefore not only causes *Bacillus anthracis* to change its Gram reaction from sharply Gram-positive to sharply Gram-negative; it also leads to a reduction, by as much as 40 per cent or even more, in its transverse diameter. Whether a similar change in longitudinal measurement also occurs it is impossible to say with certainty since the variation in length of the individuals in a given smear of *B. anthracis* is usually so great as to make comparative measurements impossible. But Gram-positive material may be shown to exist between the Gram-negative segments of a chain of *B. anthracis* and when this is removed from the ends of the organisms by excessive decolorization of lightly stained specimens diminution in length must occur (see Fig. 4, *A*, *a*, *b*, and *c*, and *C*, *a* and *b*).

Significance of Reversal.

What is the significance of the change in Gram reaction and the diminution in size produced by the exposure of *B. anthracis* to acriviolet? It is clearly not a specific reaction since it is produced also by substances other than dyes and indeed occurs in a few individuals even in the control tubes. Since acriviolet is known to have a marked bacteriostatic effect on *B. anthracis*, the reversal produced by this dye might be thought to be purely a death phenomenon. This, however, seems unlikely to be the case since other organisms (like *M. freundreichii*) which are killed by acriviolet do not exhibit reversal on exposure to this dye, and since—if the vegetative forms of *B. anthracis* be killed in other ways (as by prolonged boiling or exposure to bichloride of mercury)—reversal is not produced. Since a certain amount of reversal usually occurs sooner or later in the control tubes to which no dye has been added, it is not unlikely that the dye simply promotes or hastens a change in the bacteria, caused by degeneration or lysis (dependent perhaps on change of reaction), which would occur without any dye. Bacteria are no longer thought of as entities

with permanent characteristics, but biological units which change constantly. It is now well known that in their cycle of growth they both lose and acquire characteristics; the Gram reaction is one of the characteristics which may change during growth or be changed by environment. No one has ever demonstrated an anatomical basis which might explain this change of Gram reaction; in the case of *B. anthracis* the finer structure of the organism as revealed by these studies appears to offer such an anatomical basis.

But what is the cause of the change in size? Three explanations suggest themselves. It might be thought that the change in size was apparent rather than real and that it resulted from the heaping up of methyl violet on the surface of the Gram-positive segments, a heaping up which did not occur in the Gram-negative segments whose diameter appeared therefore to be less. It is possible of course that the apparent size of the stained bacteria is greater than the actual size of the living organisms but I am inclined to think that the difference in size produced by staining is not great. In any case the suggestion that the observed difference in size between Gram-negative and Gram-positive segments is not real but entirely dependent on accumulations of stain in some segments which do not occur in others is excluded by the fact that the difference in size is to be observed (under the limitation noted below) also in hanging drop specimens—which have not been stained at all—as well as in specimens stained by safranin alone, by fuchsin alone, by Burke's methyl violet alone, and by saturated alcoholic gentian violet alone (Fig. 3, C and D).

The second explanation which suggests itself is that the difference in size between adjacent Gram-positive and Gram-negative segments is due to shrinkage. It is conceivable that when dye is added to an aqueous suspension of *B. anthracis* osmotic forces, acting through the bacterial membrane, extract material (possibly water) from certain segments, whose size is in this way diminished. Since the weighing experiments were done with desiccated specimens, dehydration could not be the cause of the loss of weight in the Gram-negative segments.

If dehydration be brought about by exposing the bacteria to 95 per cent, followed by absolute alcohol no such picture is obtained as follows exposure to the

dyes. A slight diminution in diameter does occur, as the following measurements show, but the Gram reaction is not reversed.

Diameter control organisms (15 measured).....	1.06 μ
“ after 48 hours in 95 per cent alcohol.....	1.00 μ
“ “ 24 “ additional absolute alcohol.....	.85 μ
<hr/>	
Total loss of diameter.....	.21 μ
	20 per cent

If a bacterial suspension be evaporated to dryness and complete desiccation thus produced smears for examination of the stony material which results can only be obtained after grinding it in a mortar. This vigorous treatment destroys bacterial morphology and no satisfactory observations can be made on the debris.

A third explanation of the phenomenon is that the difference in size observed in the hanging drop as well as in stained specimens is a real difference and that it is to be explained by the fact that *B. anthracis* is composed of two parts, an outer layer which is Gram-positive, and an inner core which is Gram-negative. According to this explanation, when the proper dye is added to a suspension of *B. anthracis* the outer Gram-positive coat is destroyed and only the inner Gram-negative core remains. Ectoplasm and endoplasm might be the most correct terms to apply to these portions of the bacterial structure if they had not already been used by Zettnow¹⁴ in a somewhat different sense. Cortex and medulla are convenient descriptive terms although usually applied to an organ rather than an organism. Adopting these terms tentatively we may say—if the suggested hypothesis as to difference in size is correct—that the Gram-positive cortex of *B. anthracis* is rather easily destroyed by lysis and by degeneration; that it undoubtedly often disappears during bacterial growth (which accounts for the occasional presence of Gram-negative forms of *B. anthracis* in smears from cultures and their frequency in old cultures); that the cortex is susceptible to the action of distilled water, so that it will disappear in this medium after prolonged exposure; that it is notably sensitive to the action of certain dyes; and that when the cortex is thus destroyed the slender Gram-negative medulla comes into view.

¹⁴ Zettnow, *Z. Hyg. u. Infektionskrankh.*, 1899, xxx, 1; 1918, lxxxv, 17.

A good deal of evidence has been gathered for the correctness of this hypothesis, but it cannot perhaps be said that the case is absolutely established. Final proof would be afforded if cross-sections of bacteria could be obtained and if these, when stained by Burke's method, exhibited a Gram-negative core and a Gram-positive periphery. This proof, though we have endeavored to obtain it, cannot now be presented at least in convincing form. Numerous efforts have been made by varying technic to get satisfactory bacterial sections but, largely because of technical difficulties, without success. In the preparations examined a few objects were seen which strongly suggested cross-sections of bacteria, pink in the centre and blue at the periphery. But in examining these preparations, with the high-power and strong illumination required, color distortions occur—particularly in looking at minute objects—and these may easily lead one astray. Further attempts of this kind are now in course.

Perhaps as strong evidence as could be desired for the hypothesis that *B. anthracis* consists of a Gram-positive cortex and a Gram-negative medulla (short of that which bacterial sections would furnish), is provided by the picture obtained when smears from a young culture of this organism are stained by a modified Burke technic in which duration of exposure to dye and mordant is greatly shortened, and duration of exposure to decolorizer greatly increased. In specimens of this kind one finds individual bacteria in all stages of decolorization. A few of them may have retained their Gram positivity. But many are entirely Gram-negative, while still others show the remains of Gram-positive material clinging to the surface in the form of granules or lumps or plaques among which the pink Gram-negative medulla can be seen shining through. The appearance is exactly that of a banana, the skin of which had been in places removed, partially exposing the fruit within (see Fig. 4, A). Such a picture fits in well with the hypothesis under discussion.* Whether in specimens which have thus been partially decolorized bacterial substance is actually dissolved away from the surface of the organism by the

* A. T. Henrici (*J. Med. Research*, 1914, xxx, 409) has described similar partial decolorizations of yeast cells but appears not to have drawn any conclusions as to the significance of these observations beyond their bearing on the Gram reaction.

decolorizer, or whether merely the mordanted methyl violet is dissolved away leaving the now unstained and invisible bacterial surface material behind, has not yet been definitely proven. The former explanation seems perhaps more likely to be the correct one. If it is, then specimens which have been partially decolorized ought—if restained by the ordinary Burke technic—to look just as they did before the restaining, since the surface material, capable of retaining the methyl violet, has been in large part removed by the partial decolorization. Such a result is illustrated in Fig. 4, C. The only difference between this picture, which represents a specimen which has been partially decolorized and then restained by Burke and the picture shown in Fig. 4, A,—which represents a specimen that has been partially decolorized but not restained—is that the Gram-negative material in Fig. 4, C, has a slight purplish tint. If such a result had been always obtained when this experiment was done the matter would be clear enough. But sometimes the partially decolorized organisms, when restained, retained the Gram stain throughout.

Two critical experiments could be done to determine whether the change of size, in the bacteria which have been exposed to dye, actually rested on the anatomical structure of *B. anthracis* as we have suggested. One ought to be able to show an actual loss of weight in the bacteria which have been subjected to the dye, as compared with the controls; the demonstration of such a loss of weight, provided the specimens be desiccated before weighing, would prove that apparent diminution in size was real and would eliminate shrinkage as an explanation of the phenomena. One ought also to be able to demonstrate the presence of protein or hydrolytic products of protein in the acriviolet to which the bacteria had been exposed. These two tests were made and since both gave definite results agreeing with the hypothesis they provided additional testimony that the explanation suggested was correct.

Weighing Experiments.

The weight of the desiccated centrifugate from a heavy aqueous suspension of *B. anthracis* was determined and compared with the weight of a similar centrifugate from a suspension to which gentian violet had been added and in which reversal of Gram and diminution

in calibre had been produced. Definite loss of weight was shown to have occurred in the gentian violet suspension. These experiments gave the following results.

	Control	Gentian violet tube
Weight of tube plus dried centrifugate...	11.350 gm.	11.8250 gm.
" " empty tube.....	11.324 "	11.8114 "
<hr/>		
" " bacteria.....	.027 "	.0136 "
	.0136 "	

Loss of weight in gentian violet tube..... .0134 " = 49.6 per cent

If it be assumed that all the gentian violet dissolved in the tube went to the bottom with the bacteria during centrifugation the figures become (since the amount of gentian violet used weighed .001 gm.):

Weight of bacteria in control tube.....	.027 gm.
" " " " gentian violet tube.....	.0126 "

Loss of weight in gentian violet tube..... .0144 " = 53.3 per cent

This experiment was repeated by identical technic except that on this occasion it was necessary to allow the tube, to which gentian violet had been added, to stand only 3 hours in order to produce a complete reversal of Gram reaction. The results of this experiment were as follows:

	Control	Gentian violet tube
Weight of tube plus dried centrifugate. . .	11.4535 gm.	11.0547 gm.
" " empty tube.....	11.3336 "	11.0274 "
<hr/>		
" " bacteria.....	.1199 "	.0273 "
	.0273 "	

Loss of weight in gentian violet tube.... .0926 " = 77.2 per cent

The experiment was again repeated in another way. Instead of centrifugating the specimens they were passed through previously weighed Berkefeld N 5 filters, which were then dried and weighed. The results were as follows:

	Control	Gentian violet tube
Weight of filter plus dried bacteria.	64.1370 gm.	74.9680 gm.
" " " alone.....	61.5384 "	73.3028 "
<hr/>		
" " bacteria.....	2.5986 "	1.6652 "
	1.6652 "	

Loss of weight in gentian violet suspension .9334 " = 35.9 + per cent

Protein Tests of the Filtrate.

If the suggestion is correct that the difference in diameter between the Gram-positive and Gram-negative forms of *B. anthracis*, is due to the solution of an outer Gram-positive coat, the presence of this protein—or protein-like—material ought to be demonstrable in the filtrate of a suspension of *B. anthracis* to which dye has been added. Such a demonstration was made. For the purpose the ninhydrin test was used. This test is not specific for proteins¹⁵⁻¹⁷ so that it can only be said—not that the presence of protein was demonstrated—but that ninhydrin-positive bodies were present in the filtrates from suspensions of *B. anthracis* which had been exposed to dyes. These experiments were done as follows:

Heavy aqueous suspensions of *B. anthracis* were centrifugated, washed three times in saline, and filtered through unused Berkefeld filters into glassware which had been cleansed with aqua regia. Filtrates were tested by ninhydrin, biuret, and Heller tests. The control filtrates were negative. The filtrates from suspensions to which dye had been added gave a positive ninhydrin test, and, when concentrated by evaporation, a positive biuret.

It seems clear therefore that material, probably but not certainly protein in nature, is dissolved away by gentian violet, since its presence can be determined in the filtrate from suspensions of *B. anthracis* to which the dye has been added, the controls (made with saline in order to avoid bacteriolysis) being negative by the biuret and ninhydrin tests. Experiments are now under way to determine the nature of the material present in the gentian violet filtrate and to see whether it has any toxic, immunizing, or other important properties.

[Many observers have noticed the occurrence, in cultures of *B. anthracis* (and indeed of other organisms), of bizarre forms. Some of these bear a remote similarity to those produced by exposure to dyes. But they have usually been dismissed as "involution forms" resulting from plasmolysis or plasmoptysis (e.g. Gotschlich),¹⁸ or as

¹⁵ Ruhemann, S., *J. Chem. Soc.*, 1910, xcvii, 2030; 1911, xcix, 798.

¹⁶ Neuberg, C., *Biochem. Z.*, 1913, lvi, 500-506; 1914, lxvii, 56.

¹⁷ Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1916, xxv, 350.

¹⁸ Gotschlich, E., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1912, i, 45.

"teratological forms,"¹⁹ or as "degeneration forms." In his beautiful studies of the morphology of *B. anthracis* Preisz²⁰ pictures, without apparently paying much attention to them, bizarre forms of this kind. He worked with old cultures, the organisms were not stained by Gram, but vitally stained with fuchsin, and nothing is said of their Gram reaction. In Fig. 7, Table 3, of his 1909 publication stout and slender segments are represented. Nothing is said about them. The specimen was not stained by Gram. It was made from a 2 days old culture which had been exposed to a mixture of dog and guinea pig serum (5:1), and Preisz appears to have paid little attention to the question of size. He made no measurements. Preisz' chief interest lay in the capsule which he regarded as a degeneration product of the cell membrane. Zettnow,¹⁴ who was interested chiefly in bacterial chromatin, worked solely with the Romanowski stain. He described the bacillus of anthrax as composed of two parts: an ectoplasm, which remains colorless by the Romanowski and all ordinary staining methods, and an entoplasm which stains blue by Romanowski, and which contains chromatin (stained red by Romanowski). "A large number of bacteria consist entirely of chromatin. Even in those which stain both blue and red, chromatin strongly predominates. In exceptional cases in very young cultures the plasma occurs in larger amounts than the chromatin but as growth proceeds the relation is reversed." The ectoplasm, compared to the entoplasm, is said to be relatively poor in water content and is more concentrated, as is clear from its greater resistance to stains, to plasmolysis, and to destructive influences. In contradistinction to *B. anthracis*, cocci and sarcinae take only one color, the blue (entoplasm). Zettnow made no studies by the Gram method. He made no measurements. In our own work smears of organisms which had been exposed to acriviolet until partial reversal had occurred were stained by numerous polychrome methods, including Romanowski. No selective staining, such as that described by Zettnow, was observed. From all these data it seems clear that there is no relation between the picture obtained by Zettnow and that described in this communication. Zettnow's ectoplasm is said to remain colorless

¹⁹ Maassen, A., *Arb. k. Gsndhtsamte*, 1904, xxi, 385.

²⁰ Preisz, H., *Centr. Bakt., 1. Abt., Orig.*, 1903-04, xxxv, 657; 1909, xlix, 341.

in all ordinary staining methods. Since both cortex and medulla stain readily both would appear to lie within the entoplasm.

Gram Stability and Gram Instability.

The fact that the material responsible for the Gram positivity of *B. anthracis* appears to be confined to the surface of this organism and that the centre is Gram-negative, made it seem likely that the Gram positivity of *B. anthracis* would be relatively unstable as compared with that, for example, of *M. freudenreichii*, an organism in which no distinction between Gram-positive surface and Gram-negative interior had been demonstrable. It had been found, indeed, by preliminary observations that,—although young cultures of *B. anthracis* were completely and definitely Gram-positive (when stained by the Burke method), provided the technic was accurately followed—an increasing proportion of individuals in a given specimen became Gram-negative if the time of exposure to dye and mordant was diminished and the time of exposure to decolorizer increased. A systematic study was therefore made to see what the effect on different Gram-positive organisms would be, of variations in time of exposure to stain, to mordant, and to decolorizer. It was found that for many organisms (*M. freudenreichii* may be cited as a typical example) variations in time of exposures made, within wide limits, practically no difference in the results, *M. freudenreichii* being always 99 per cent to 100 per cent positive. In the case of other Gram-positive organisms (*B. anthracis* for example), wide variations in the result could be obtained by varying the technic as regards time of exposure.²¹

The facts are well represented in Fig. 4, *B*, in which are illustrated the results of an experiment done with a suspension containing a mixture of *B. anthracis* (N. Y. B. of H.) and *M. freudenreichii*. The smear has been stained by a modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 10 minutes). *B. anthracis* has been largely decolorized: *M. freudenreichii* unaffected.

When investigated in two ways therefore—by the results of exposure to acriviolet (see Fig. 2) or by modifications of staining tech-

²¹ Churchman, J. W., *Stain Technol.*, 1927, ii, 21.

nic in the direction of diminished time of exposure to dye and mordant and prolonged time of exposure to decolorizer (see Fig. 4, *B*)—two Gram-positive organisms are seen to behave quite differently, as regards their Gram reaction. Light is thus thrown on the mechanism of the Gram reaction and the fact emerges that this mechanism is not necessarily the same in the case of bacteria of different species even though their behavior to the standard technic is identical. This distinction between stable and unstable Gram-positives appears to be a useful one. It is suggested that in recording the Gram reaction of new species, some such set of comparative tests as here described be carried out so that the stability of the reaction be known.

Examination of Hanging Drop Specimens.

Attention has been called to the facts that the difference in size between Gram-positive and Gram-negative segments of *B. anthracis* is demonstrable in unstained hanging drop specimens, made from suspensions which have been exposed to acriviolet or gentian violet and in smears stained with a single dye like methyl violet. From these facts—represented in Fig. 3, *C* and *D*,—one can only conclude that the reduction in calibre is real and not the result of an artefact.

The experiment of May 25, from which Fig. 3, *D*, was made, may be cited. A suspension of a 3 hour culture of *B. anthracis* No. 10 was made and gentian violet added. At the end of 2 hours smears stained by Burke, showed that the organism had changed from 100 per cent Gram-positive to 50 per cent Gram-positive and beautiful instances of great variation in size between adjacent bacterial segments were numerous. A hanging drop specimen was made. The organisms had picked up enough of the gentian violet to which they had been exposed to be clearly visible and the marked differences in size represented in the figure were readily made out. It was noticeable that the stout segments were always more deeply stained than the slender.

Observations of this sort left no doubt as to the reality of the difference in size between adjacent bacterial segments. But throughout the experiments with hanging drop specimens I was struck by a curious fact: If specimens of *B. anthracis* which had been

treated with acriviolet were stained by Burke or by Paltauf and the stout and slender forms measured, a difference of diameter—often as high as 60 per cent—was readily made out. If, however, specimens from the same tube were stained with a single dye or examined in the hanging drop this difference in size was sometimes by no means so readily observed. This observation led us at first to the conclusion that the difference in diameter which we had seen in the smears stained by Burke was apparent and not real. The demonstration, however, of an actual loss of weight, of a clear difference of size in many of the hanging drop specimens, of the presence of protein (or related substance) in the filtrate from bacterial suspensions containing acriviolet, and of the presence of cortex and medulla, by methods of partial decolorization, all pointed to a real and not an apparent loss of calibre.

The fact that the difference in size between Gram-positive and Gram-negative segments is not as a rule so obvious, in the fresh specimen and in those stained with a single stain, as it is in the specimen stained by Burke, is to be explained partly by the fact that the Burke stain gives a contrast of color which at once attracts the eye and at the same time emphasizes the difference in size. This contrast is absent in the single stains and change in size unless very marked is easily overlooked even when it is present. Another reason for the apparent discrepancy is that the Gram-negative segments—that is to say, the smaller segments—often stain (when only single stains are used) very lightly, in some cases not at all; and these ghosts easily escape detection. In the fresh specimen the slender segments are translucent, often nearly invisible, and it is probable that many of them are overlooked. It may also be the case that the Burke stain somewhat exaggerates the degree of the difference in size, which is none the less real.

It results from all this that one must have for examination in the fresh or with single stains, specimens in which the difference in size is quite marked indeed. In specimens stained by Burke on the other hand the difference in size is very striking even when it is not great in degree.

SUMMARY.

1. The addition of small amounts of aqueous gentian violet, acriflavine, or acriviolet to suspensions of young cultures of *B. anthracis* reverses their Gram reaction, and diminishes their diameter about 40 per cent.

2. The time required for these changes varies with the strain of *B. anthracis* examined.

3. These changes are accompanied by a loss of weight.

4. Ninhydrin-positive substances are demonstrable in the filtrate from suspensions of *B. anthracis* to which dyes have been added.

5. Similar changes are produced by these dyes in many, but not in all, of the sporogenic aerobes.

6. Non-spore bearers are for the most part unaffected in these ways by the dyes, although to this statement there are a number of exceptions.

7. The change in size produced by the dyes is demonstrable in hanging drop specimens as well as in stained smears, but not with equal constancy.

8. Partial decolorizations of *B. anthracis* are described, which are produced by modifications of the Burke technic in which time of exposure to dye is shortened and time of exposure to decolorizer lengthened.

9. The explanation for these phenomena which accords with all the known facts is that they depend on the existence in *B. anthracis* of a Gram-positive cortex and a Gram-negative medulla. Positive proof of the correctness of this explanation must await the evidence furnished by cross-sections of bacteria.

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Camera lucida drawing. Magnification $\times 4200$. This plate shows two successive stages in the change produced in the Gram reaction and in the calibre of *B. anthracis* (A. T. C. No. 10) by exposure to acriviolet. Smears stained by Burke's method.

A, smear made for control at the beginning of the experiment. The organism is completely Gram-positive.

B, smear made from a specimen which has been exposed to acriviolet for 45 minutes. Partial reversal of the Gram reaction has occurred, about 25 per cent

of the individuals being Gram-positive and 75 per cent Gram-negative. Notice the marked difference in calibre between the Gram-negative forms and the Gram-positive, and the fact that the spore is entirely contained in the Gram-negative portion of the organism (a). One gets the impression of a smear made from a mixture of two different organisms.

C, smear made from a specimen which has been exposed to acriviolet for 2 hours, showing complete reversal of Gram reaction. Practically every individual is now Gram-negative.

PLATE 35.

FIG. 2. Camera lucida drawing of a mixture of *B. anthracis* (N. Y. B. of H.) and *M. freudenreichii* (A. T. C.). Magnification $\times 4200$. Stained by Burke's method.

A, smear from the control tube. Both organisms are sharply and completely Gram-positive. One stray Gram-negative individual of *B. anthracis* is seen.

B, smear from specimen which has been exposed to acriviolet. The Gram reaction of *B. anthracis* has been in large part reversed, almost all of the individual organisms being now Gram-negative. All the individual organisms of *M. freudenreichii* are, on the other hand, as strongly Gram-positive as at the start of the experiment. The difference in calibre between Gram-positive and Gram-negative forms is very evident.

PLATE 36.

FIG. 3. Camera lucida drawings. Magnification $\times 4200$.

This plate represents the slight variations in difference of size, between Gram-positive and Gram-negative forms, produced by varying the conditions of the experiment.

A, smear made from a specimen of *B. anthracis* (N. Y. B. of H.) which has been exposed to acriviolet until partial reversal of Gram reaction has occurred, and stained by Paltauf's modification. The difference in calibre, between Gram-negative and Gram-positive forms, though definite, is less marked than in B. Notice (a) the spores within the medulla.

B, smear made from a specimen of *B. anthracis* (A. T. C. No. 10) which has been exposed to acriviolet and stained by Burke's modification. Compare the difference in calibre with that shown in A.

C, smear made from a specimen of *B. anthracis* (A. T. C. No. 10) which has been exposed to acriviolet and stained only with Burke's methyl violet. The presence of stout and slender forms is evident. The picture obtained in the specimens stained by the Burke technic cannot therefore be an artefact produced by that technic.

D, drawing of individual organisms seen in a hanging drop specimen made from a suspension of *B. anthracis* (A. T. C. No. 10) which has been exposed to gentian violet. The living organisms have taken up enough of the stain to make them clearly visible and the difference in calibre between Gram-positive segments

(which take the stain fairly well) and the Gram-negative segments (which remain pale) is perfectly clear. The difference in size caused by exposure to dyes is therefore real and not an artefact produced by the Burke technic.

PLATE 37.

FIG. 4. Camera lucida drawings. Magnification $\times 4200$.

A, partial decolorization of *B. anthracis* (A. T. C. No. 10) by modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 3 minutes).

The Gram-positive material has been partially removed from the surface, persisting only as plaques or lumps or stippling through which the pink Gram-negative rod in the interior of the organism can be seen. Notice (*a, b, c*) the caps of Gram-positive material at the ends of the bacterial segments.

B, smear of a mixture of *B. anthracis* (A. T. C. No. 10) and *M. freudenreichii* (A. T. C.) which has been stained by modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 10 minutes).

Almost every individual organism of *B. anthracis* has been completely decolorized; but *M. freudenreichii* has remained completely Gram-positive.

C, smear of *B. anthracis* (A. T. C. No. 10) stained by the same modification of Burke's technic as that used in the experiment represented in *A*, and then restained by the ordinary Burke technic. The portions of the organisms which were decolorized by the first process now fail to take the stain in the second and appear as in *A*, except that the pink color has become purplish pink. Notice (*a, b*) terminal caps of Gram-positive material.

PLATE 38.

FIG. 5. Photographs of single chains from specimens of *B. anthracis*, which have been exposed to acriviolet, to show the difference in calibre between Gram-negative and Gram-positive segments as seen at various magnifications. On account of the difference in calibre accurate focussing at the higher magnifications is impossible.

A, magnification $\times 1400$. Stained by Burke's modification. Alternating Gram-positive and Gram-negative segments. *B. anthracis* (A. T. C. No. 10).

B, magnification $\times 1800$. A Gram-negative segment with a Gram-positive segment at either end is plainly seen. *B. anthracis* (A. T. C. No. 10) stained by Burke's modification.

C, magnification $\times 2500$. The same chain represented in *B*.

D, magnification $\times 3200$. *B. anthracis* (N. Y. B. of H.) stained by Paltauf's modification. The difference in calibre between Gram-positive and Gram-negative segments, though clear, is less marked than in *A, B*, and *C*.

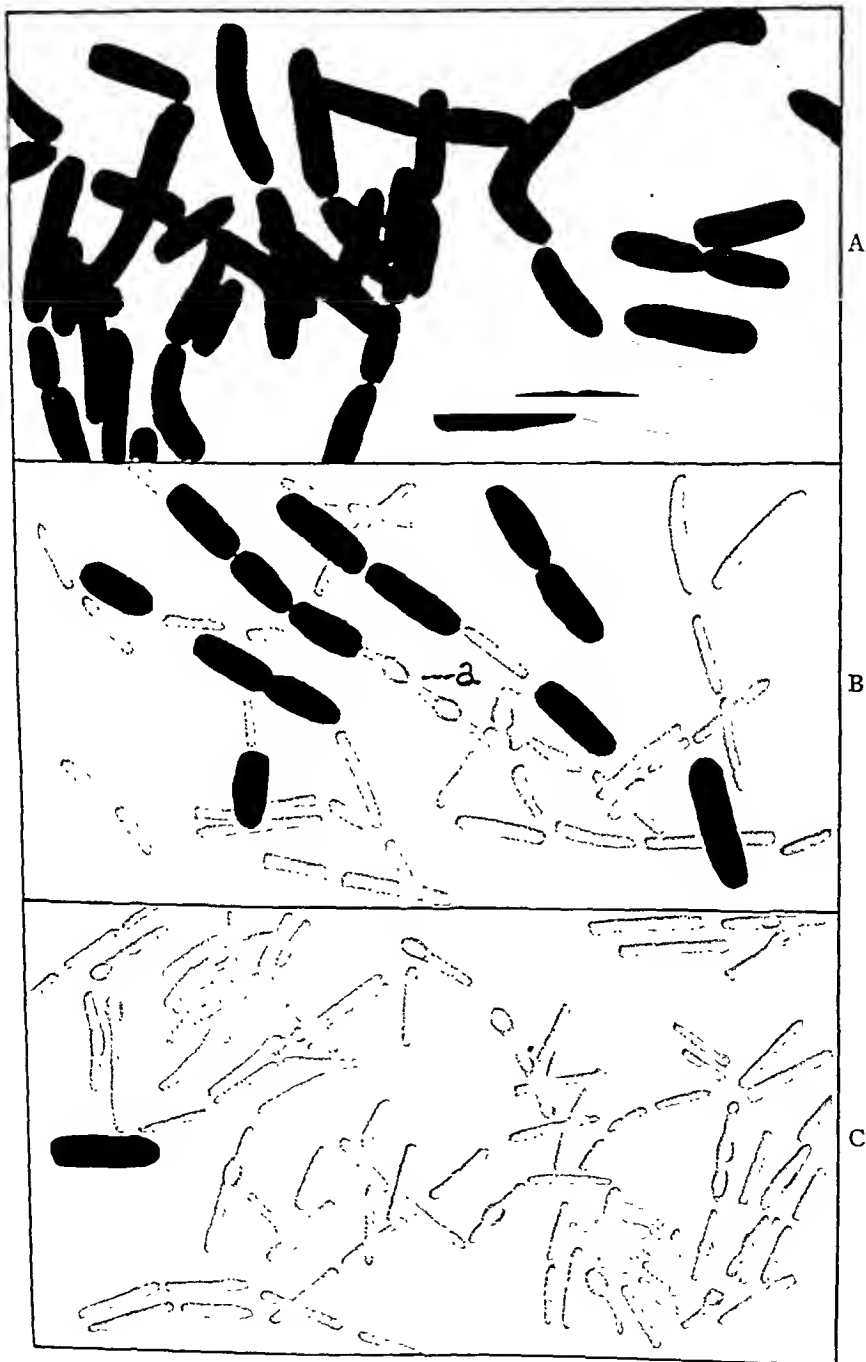
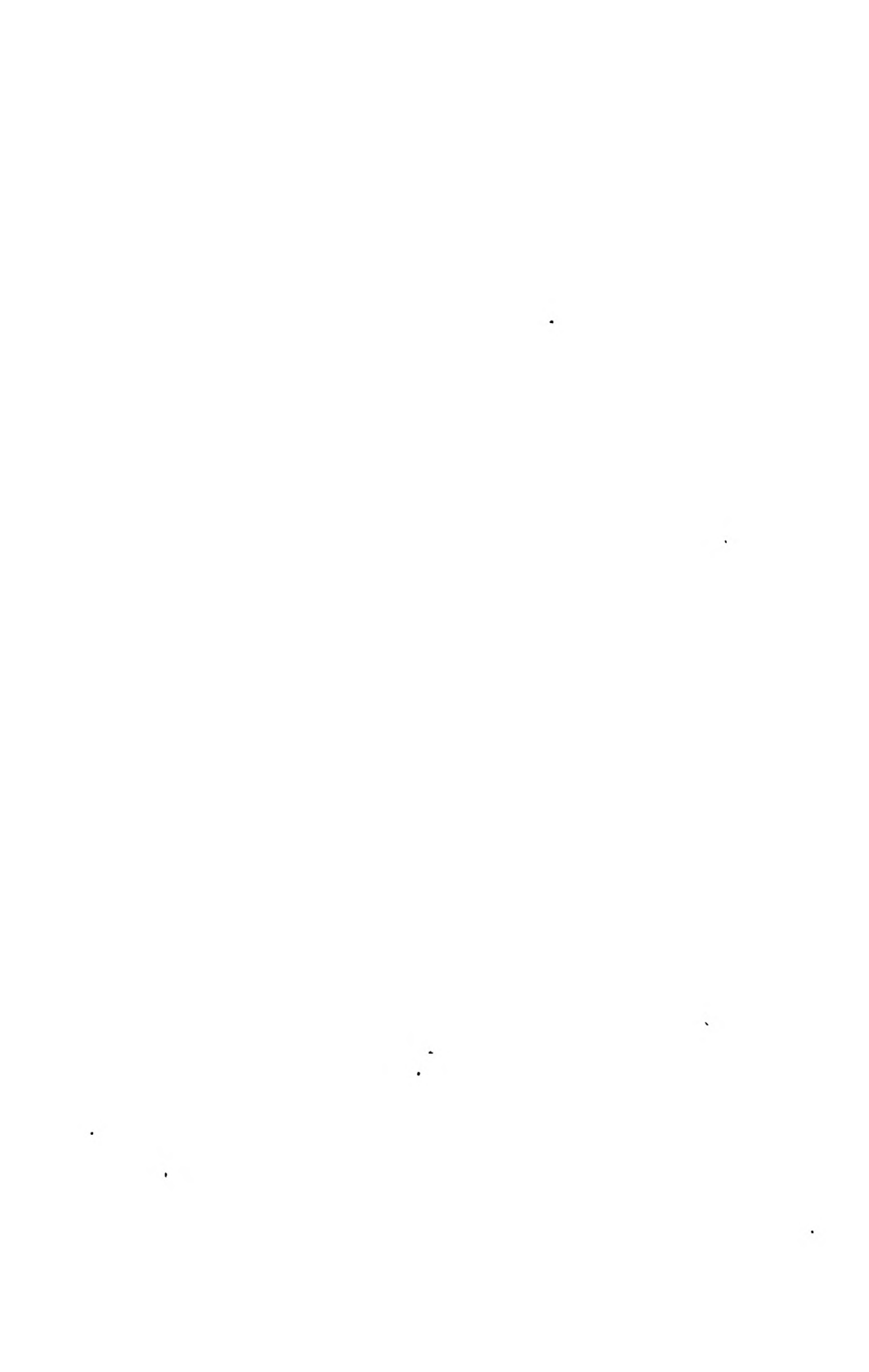


FIG. 1.

(Churchman: *B. anthracis* and Gram reaction.)



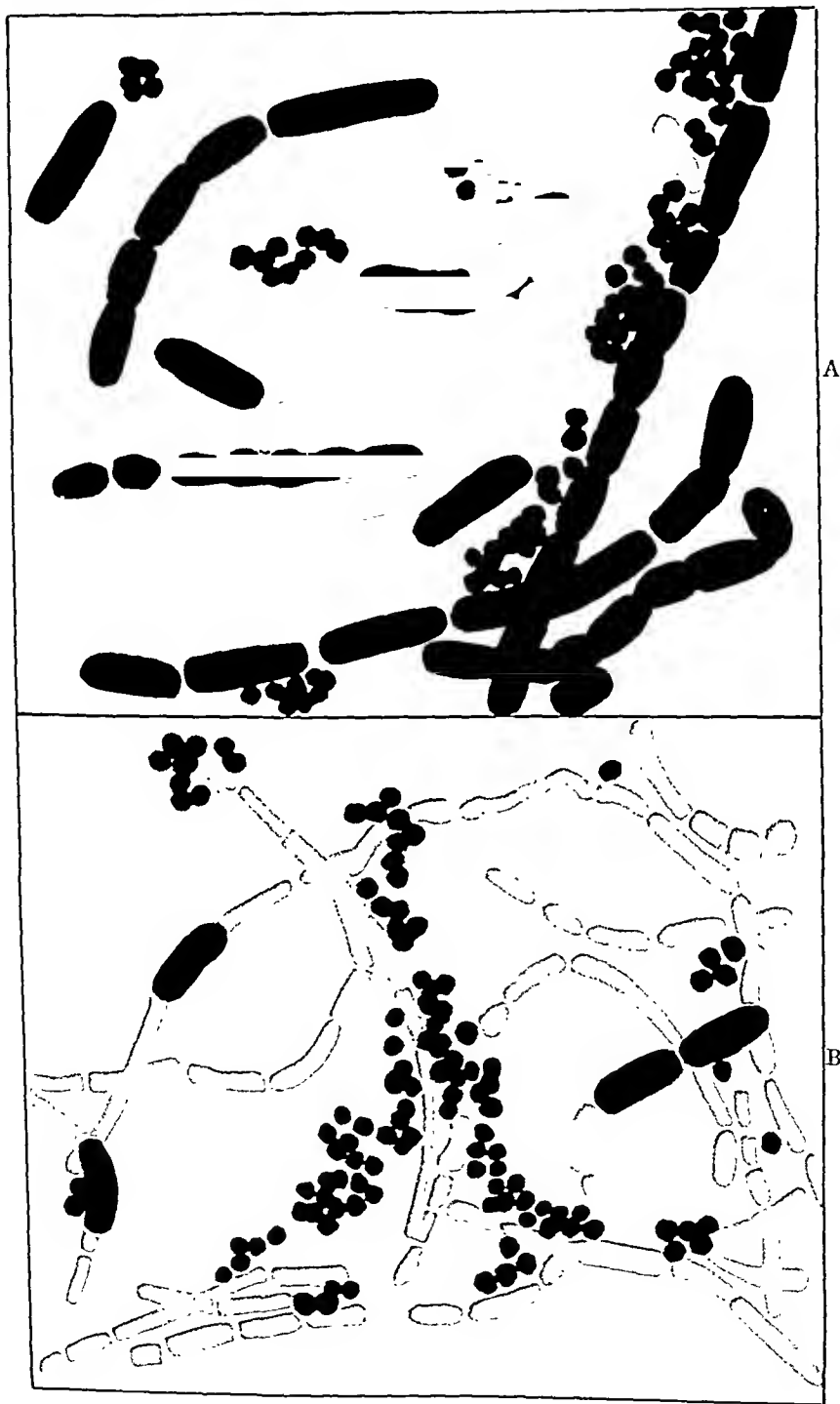


FIG. 2.

(Churchman: *B. anthracis* and Gram reaction.)

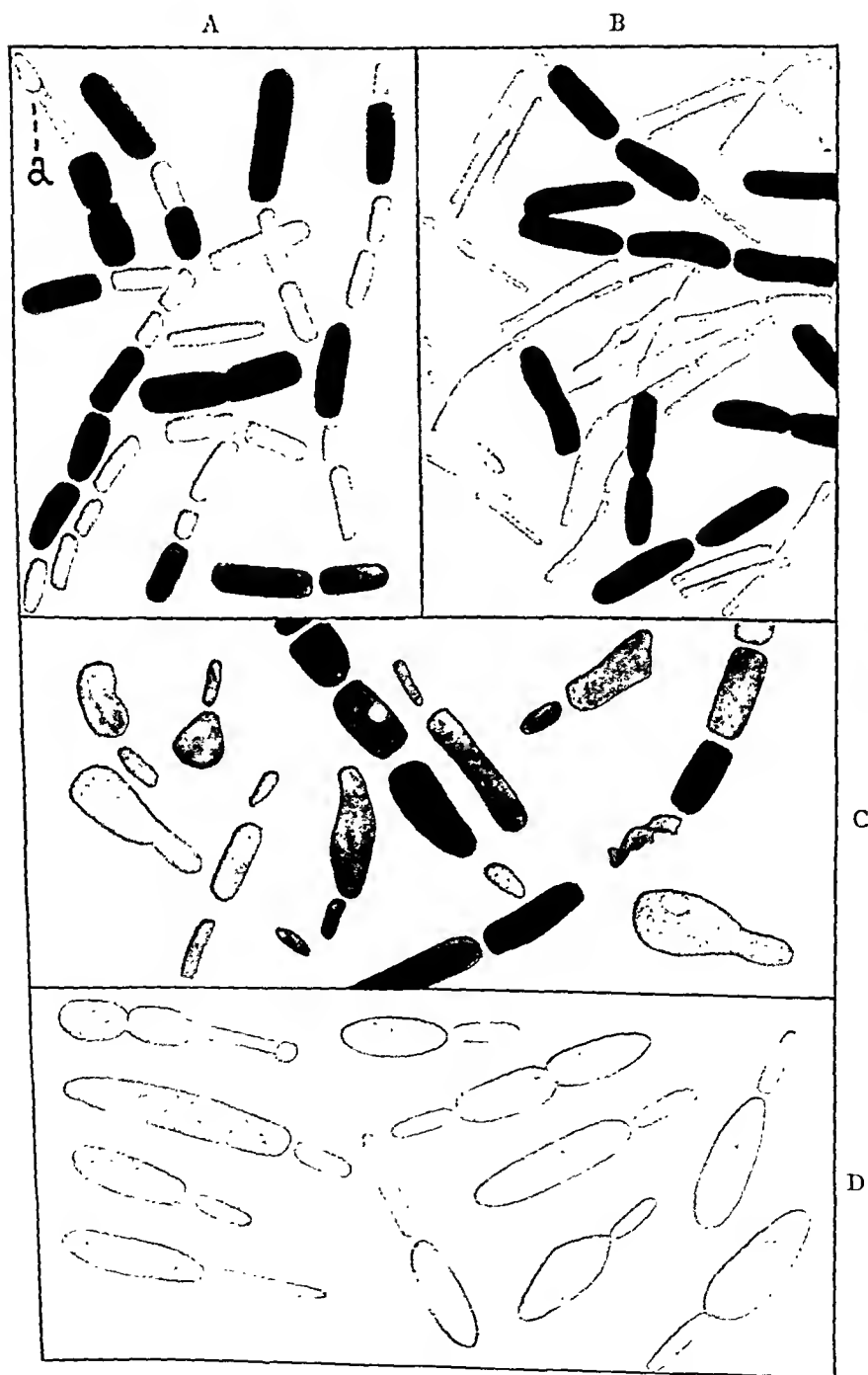


FIG. 3.

(Churchman: *B. anthracis* and Gram reaction.)

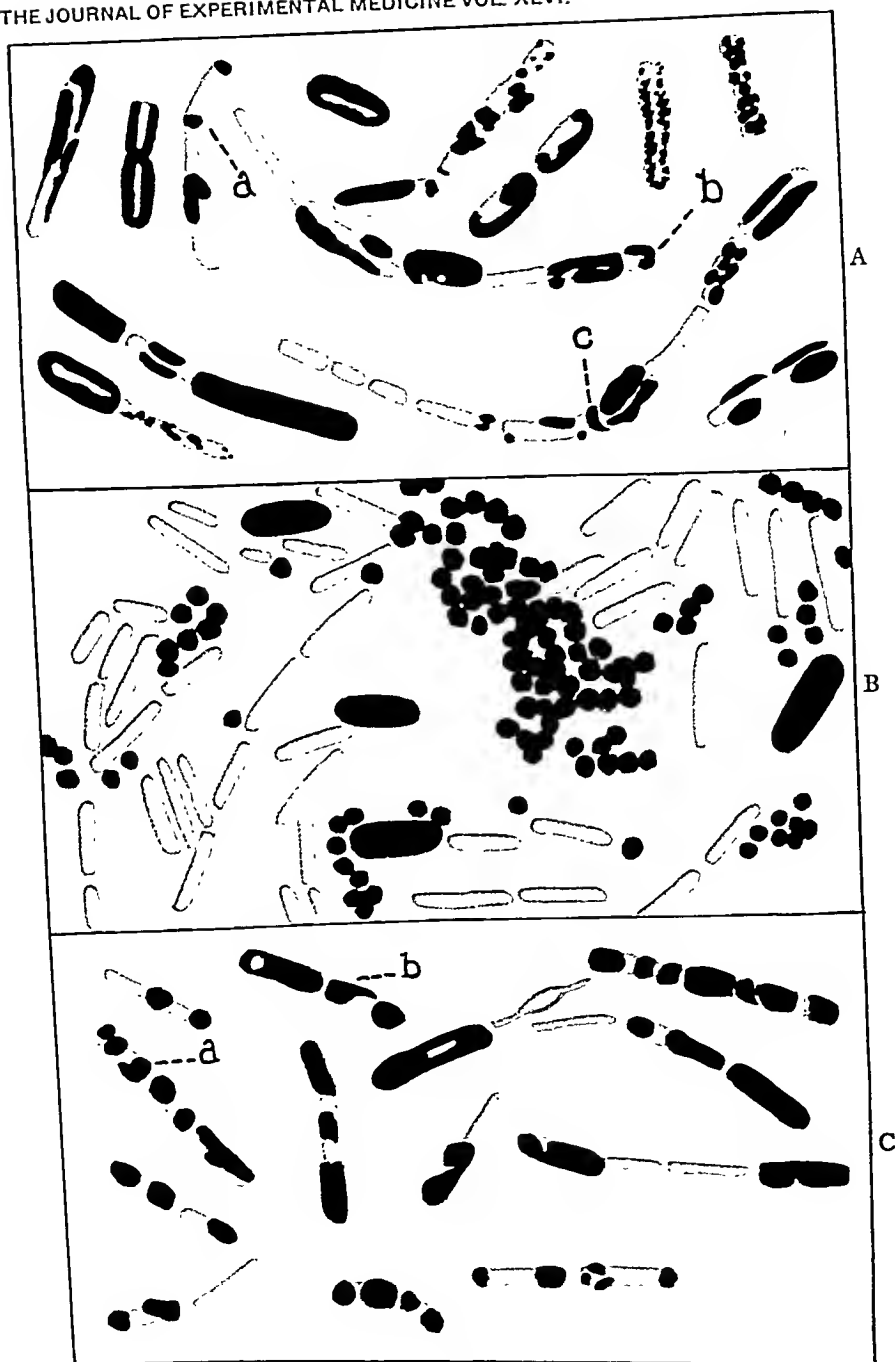


FIG. 4.

(Churchman: *B. anthracis* and Gram reaction.)



FIG. 5.

(Churchman: *B. anthracis* and Gram reaction.)

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